COORDINATING DIFFERENTIATION WITH BEHAVIORAL OUTPUT OF THE CCAP-NEURON NETWORK IN *DROSOPHILA MELANOGASTER*

by

LYUBOV VEVERYTSA

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ABSTRACT

Appropriate integration of neurons into functioning networks is the ultimate goal of neuronal differentiation. My thesis examined the mechanism and timing of neuronal differentiation in *Drosophila melanogaster* in relation to the functional requirements of a developing neuronal network.

Specifically, my thesis aimed to address the role of extrinsic signaling in inducing and maintaining the expression of genes important to the function of neurons within their network. CCAP-neurons were chosen as a model because: 1) GAL4 drivers are available for cell-specific genetic manipulation. 2) The critical role of CCAP-neurons in the behavior, ecdysis, provides for an easily assayed phenotype if these neurons fail to function properly. 3) Four peptide hormones are selectively expressed in differentiated CCAP-neurons that are essential for the normal function of CCAP-neurons in ecdysis; this provides a direct link between gene expression and behavior. 4) Ecdysis is reiterated at multiple developmental steps, thus the CCAP-neuronal population functions throughout development. Together, these factors allow my work to relate neuronal subtype-specific differentiation to the regulation of gene expression and then directly to behavior.

Larval *Drosophila* CCAP-neurons comprise ~46 neurons [~36 interneurons (CCAP-INs) and 10 efferent-neurons (CCAP-ENs)] that express a number of terminal differentiation genes (TDGs; such as neuropeptides). To begin, we delineated mechanisms underlying the expression of four TDGs, the peptide hormones CCAP, MIP, Bursicon-α and Bursicon-β, which together mediate the functional output of those neurons. Importantly, my studies found that a specific subset of CCAP-neurons, the CCAP-ENs, is both necessary and sufficient for ecdysis, and that their function in ecdysis is mediated by extrinsic BMP-dependent peptide hormone expression. Additionally, we found that the change in the ecdysis behavioral sequence from larval to pupal ecdysis is supported by the recruitment of a 'late' subset of CCAP-neurons that are born in the embryo but undergo extrinsic ecdysone-triggered, temporally-tuned differentiation immediately prior to pupal ecdysis.

PREFACE

The work in chapter 2, entitled "Retrograde BMP-signaling controls *Drosophila* behavior through regulation of a peptide hormone battery**" has been published as:**

Veverytsa, L and Allan, D.W. 2011. Retrograde BMP-signaling controls *Drosophila* behavior through regulation of a peptide hormone battery. *Development*. Aug;138 (15):3147-57.

The work in chapter 3, entitled "Temporally-tuned differentiation of a *Drosophila* CCAP-neuron subset remodels a functioning network to support a behavioral switch**" has been submitted for publication as:**

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DWA and LV conceived all experiments. LV conducted all experiments and data analysis. DWA and LV wrote both manuscripts. LV wrote the thesis.

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5-HT	5- hydroxytryptamine
20E	20--hydroxyecdysone
APF	After puparium formation
BMP	Bone morphogenetic protein
Brk	Brinker
BRE	BMP-response element
Bursα	Bursiconα
Bursβ	Bursiconβ
CCAP	Cardioacceleratory peptide
CGRP	Calcitonin-gene-related peptide
CNS	central nervous system
Dac	Dachshund
Dad	Daughters against dpp
DN	Dominant negative
Dpp	Decapentaplegic
EcR-A/B1/B2	Ecdysone receptor A/B1/B2
EH	Eclosion hormone
EN	Efferent neuron
ETH	Ecdysis triggering hormone
Flp	Flippase
FRT	Flippase recognition target
Ftz-f1	Fushi-tarazu transcription factor1
Gbb	Glass bottom boat
GDNF	Glial-cell-line-derived neurotrophic factor
GMC	Ganglion mother cell
hs	Heat shock promoter
IN	interneuron
Kir2.1	inwardly rectifying K^+ channel
L.1	larval stage
Lack	Lethal with a checkpoint kinase
LBD	Ligand-binding domain

LTF	Long-term facilitation
Mad	Mothers against decapentaplegic
MIP	Myoinhibiting peptide precursor
NB	Neuroblast
NGF	Nerve growth factor
NHR	Nuclear hormone receptor
NSD	No significant difference
NT-3	Neurotrohin3
PA	Pharate adult
PACAP	Pituitary adenylate cyclase-activating polypeptide
Sax	Saxophone
Shn	Shnurri
Sog	Short gastrulation
TDG	Terminal differentiation gene
Tkv	thickveins
TF	Transcription factor
Tsg	Twisted gastrulation
Usp	Ultraspiracle
VGluT	Glutamate vesicular transporter
VIP	Vasoactive interstinal peptide
VNC	Ventral nerve cord
Wit	wishful thinking

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DEDICATION

Dedicated to my parents, who made great sacrifices so I could fulfill my dreams.

1. INTRODUCTION

1.1 NEURONAL DIFFERENTIATION

A great diversity of neurons is generated during nervous system development. Neuronal diversity is due to differences in the expression of batteries of 'terminal differentiation genes' (TDGs) that define each neuron's unique identity and function (neuropeptides, ion channels, neurotransmitter synthetic enzymes, receptors, axon guidance molecules etc) (Hippenmeyer et al., 2004; Nishi, 2003; Sisk and Foster, 2004; Smith et al., 2006; Spitzer et al., 2005; Xu and Hall, 2006). How does this great diversity of neuronal subtypes arise? Great progress has been made to elucidate the genetic mechanisms underlying the generation of cell diversity in the nervous system. It has been proposed that cell-specific combinations of transcription factors (TFs) and 'target-derived' signals determine which TDGs are expressed in neurons (Briscoe and Novitch, 2008; di Sanguinetto et al., 2008; Lee and Pfaff, 2001; Shirasaki and Pfaff, 2002).

1.1.1. Specification and differentiation of neurons

During early *Drosophila* embryogenesis, complex patterning processes along the antero-posterior and dorso-ventral axes establish a "grid" of morphogen and transcription factor (TF) expression within the embryo, including the emerging neuroectoderm (Karcavich, 2005). *Drosophila* neuroblasts (NBs) then delaminate from the neuroectoderm with unique combinatorial codes of TFs, encoded by the patterning processes of early development (Skeath and Thor, 2003). *Drosophila* NBs undergo a (mostly) invariant series of asymmetric cell divisions, each time generating a neuroblast and a ganglion mother cell (GMC). Each GMC subsequently divides to generate a pair of neurons and/or glia. The series of NB and GMC divisions within numerous NB lineages have been defined (Pearson and Doe, 2004). After each NB division, most NBs switch through a series of 'temporal code' TFs in the following sequence: Hunchback → Kruppel → Pdm (POU domain TF) → Castor → Grainy head. Studies on variability between lineages showed that many different NB lineages utilize this same temporal code (Brody and Odenwald, 2000; Pearson and Doe, 2004). Through this process,

spatial and temporal TF combinatorial codes play a role in specifying the diversity of neuronal subtypes that arise in the central nervous system.

These spatial and temporal TF codes specify the fate of newborn postmitotic neurons through activating the expression of cell-specific combinations of TFs in postmitotic neurons. These postmitotic combinations of TFs are then combinatorially required for terminal differentiation. How do such TF codes act in postmitotic neurons to induce the expression of subtype-specific TDG to differentiate subtype identity? Surprisingly, although we know many TFs that play a role in subtype-specific differentiation, we still have a poor understanding of how TF codes generate subtype-specific programs of gene expression. Subtype identity is not defined by a single unique TDG. Rather, it is the unique combination of TDGs. The only systematic effort to understand how TFs coordinate the gene expression in postmitotic neurons was performed in *C. elegans* (Etchberger et al., 2007; Flames and Hobert, 2009; Hobert, 2008; Wenick and Hobert, 2004). These studies found that most genes in neurons are activated by just one or two TFs acting cooperatively in a cell-specific manner. However, the nervous system of *C. elegans* has only 302 neurons and an estimated 950 TFs in its genome, which would permit a certain simplicity in coding for neuronal diversity. In organisms that have many more neurons than TFs, such as *Drosophila* (100,000 neurons and 750 TFs) or mouse (100 million neurons and 1500 TFs), a ration of one TF to one neuron cannot explain neuronal diversity. Our laboratory and others have shown that in metazoans with increased neuronal complexity, many more TFs are required combinatorially to activate any single TDG cell specifically (Allan et al., 2005; Jorgensen et al., 2004). Moreover, we and others have shown that different combinations of TFs act within single neurons to *trans*-activate specific subsets of TDGs (Allan et al., 2005; Jorgensen et al., 2004; Miguel-Aliaga et al., 2004). Understanding how these TF programs coordinate diversified profiles of gene expression in the neurons of metazoans with more complex nervous systems remains an important challenge.

1.2 ROLE OF TARGET-DERIVED SIGNALS IN NEURONAL DIFFERENTIATION

In both vertebrates and *Drosophila*, terminal differentiation of neurons does not depend solely on the combination of TFs. Target-derived retrograde signals such as BMPs, activins, cytokines and neurotrophins, secreted from neuronal targets, further influence neuronal differentiation, most notably in genes responsible for neuronal communication, such as neuropeptides and neurotransmitter synthetic enzymes (da Silva and Wang, 2011; Ernsberger

and Rohrer, 1999; Hippenmeyer et al., 2004; Nishi, 2003; Schotzinger et al., 1994; Xu and Hall, 2006). Such retrograde signals are also essential for neuronal survival as well as synaptic formation and plasticity (Ernsberger, 2009; Huang and Reichardt, 2001). Retrograde signaling involves interaction of a ligand from the postsynaptic cell, and receptor-mediated signal transduction in the presynaptic neuron. These signals can act locally to influence synaptic function and can be retrogradely trafficked to the nucleus to regulate gene expression. The first retrograde signal discovered was the neurotrophin, nerve growth factor (NGF). NGF and other neurotrophins are typically produced by the targets of neurons and are required for neuronal survival and growth. Dr. Levi-Montalcini discovered NGF while looking for survival factors for chick sensory and sympathetic neurons (Ernsberger and Rohrer, 1999; Fitzsimonds and Poo, 1998; Ladle et al., 2007). The mechanism by which NGF and other neurotrophins are transported has been thoroughly analyzed and found to involve retrograde trafficking of signaling endosomes. First, neurotrophins bind cognate receptors on the presynaptic surface. This induces local endocytosis and transport of ligand/receptor complexes. Continuous ligand/receptor interaction within the signaling endosome then ensures persistent signal transduction from the synapse to the cell body (Campenot and MacInnis, 2004; Howe and Mobley, 2005; Ibanez, 2007; Jordan and Kreutz, 2009; Zweifel et al., 2005).

Story Landis' studies were the first to discover that target-derived signaling influences neuronal differentiation. Landis showed that the footpad sweat glands of rodents are initially innervated by noradrenergic sympathetic axons. However, over time this innervation loses its noradrenergic properties to become cholinergic. Key evidence for a target-derived signal inducing this switch included transplanting a mature footpad to the skin of the lower thoracic region of a neonatal rat. Remarkably, the noradrenergic sympathetic neurons that start to innervate the transplanted footpad became cholinergic (Schotzinger and Landis, 1988). In contrast, transplanted parotid gland (innervated by noradrenergic sympathetic neurons) did not induce cholinergic properties in sympathetic neurons. Finally, Landis showed that mice that lack sweat glands do not have cholinergic fibers in their footpad. Thus, the sweat gland is the inductive target that induces sympathetic cholinergic properties (Ernsberger and Rohrer, 1999; Schotzinger and Landis, 1988).

Since then, numerous examples of target-derived signaling have been uncovered, using neurotrophins, cytokines or TGFβ family pathways as retrograde signal (da Silva and Wang, 2011; Hippenmeyer et al., 2004; Nishi, 2003). In this section I will briefly discuss the roles of neurotrophin signaling in retrograde neuronal differentiation. However, because TGFβ/BMP

signaling is a major focus for my thesis, I will discuss this in a more detail in the following section. Brachial motoneurons express the ETS-type TF Pea3 in response to muscle target-derived glial-cell-line-derived neurotrophic factor (GDNF). Pea3 is in turn required for proper muscle innervation, dendritic connectivity and motor pool clustering (Vrieseling and Arber, 2006). Similarly, proprioceptive Ia afferents receive neurotrophin3 (NT-3) signaling from their target muscle spindles. This induces expression of the ETS-type TF, Er81, which is then required for proper central connectivity onto motoneurons (Patel et al., 2003). Cutaneous afferents target the skin during development. They receive NGF and activin (TGFβ-family) signaling from the skin, which are both required for induction of calcitonin-gene-related peptide (CGRP) expression (Xu and Hall, 2007).

1.3 THE BMP PATHWAY

The BMP pathway has emerged as a major mediator of retrograde signaling, and is currently the only pathway known to play a conserved role from *Drosophila* to vertebrates in retrograde neuronal differentiation (Allan et al., 2003; Hodge et al., 2007). Bone Morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily of morphogens that have a conserved role in target-derived retrograde signaling in neurons (Fig.1). BMPs are involved in a variety of cellular functions including osteogenesis, cell differentiation, growth, homeostasis and axial patterning of embryos (Cao and Chen, 2005; Kitisin et al., 2007; Liu and Niswander, 2005). BMP-type activity was first identified in the 1960's and proteins responsible for osteogenesis were identified in the 1980's (Urist, 1965; Wozney et al., 1988). Currently over twenty members of the BMP family have been identified in mammals (Kishigami and Mishina, 2005). The BMP signal transduction pathway and its regulators are highly conserved between invertebrates and vertebrates (Fig.2).

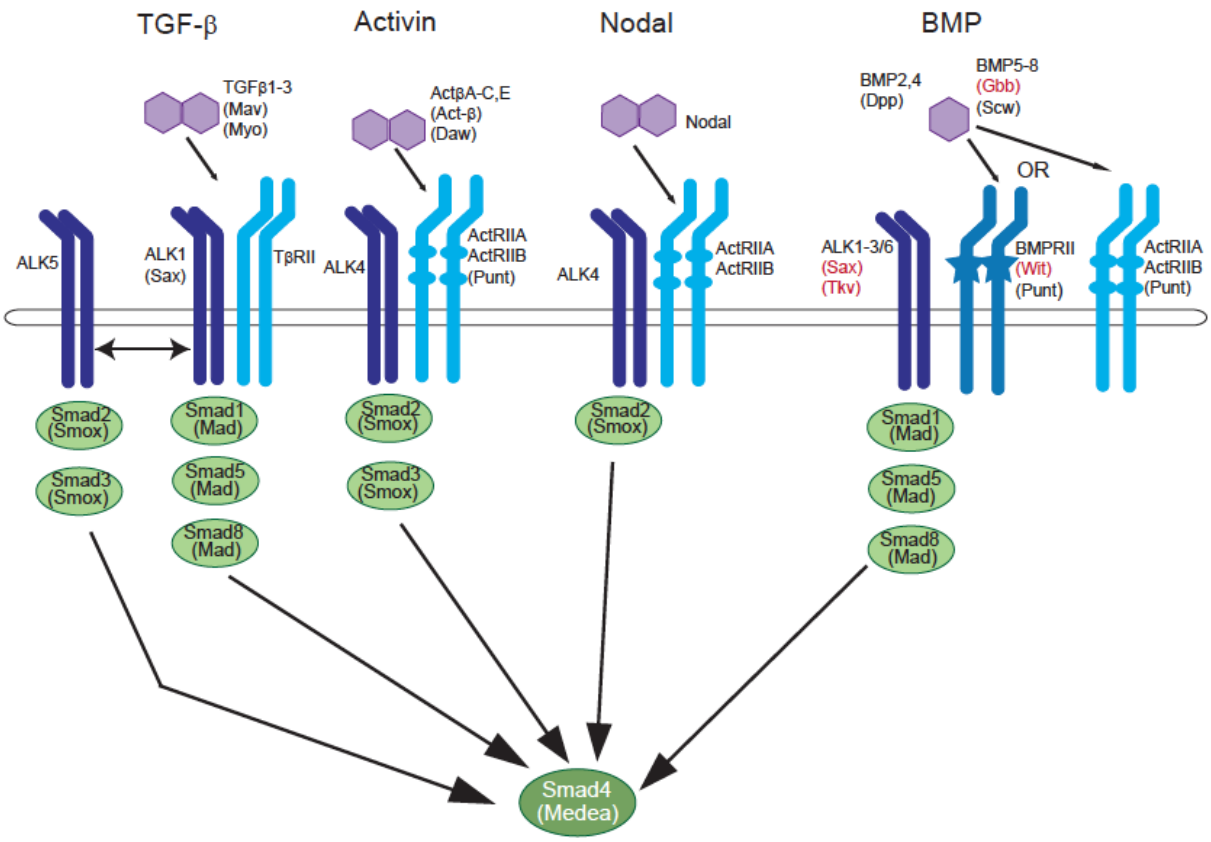


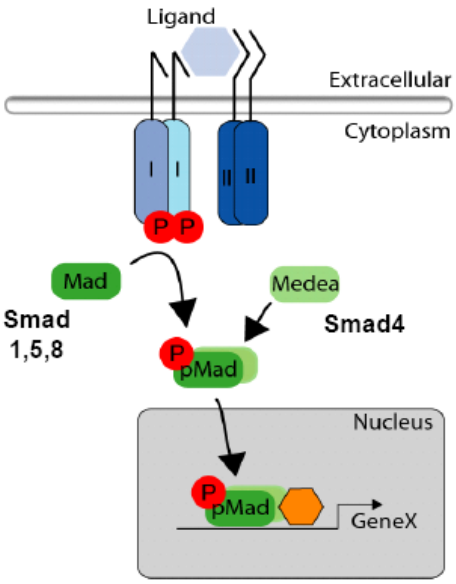
Figure 1.1 Transforming growth factor-β superfamily

TGF-β superfamily includes TGF-β, Activin, Nodal and BMP pathways. Mouse components are labeled beside the cognate ligand/receptor/Smad. All known *Drosophila* components of each of these pathways are shown in brackets. BMP pathway components that play a role in *Drosophila* neurons are written in red. (Image made by Lyubov Veverytsa)

BMP ligands engage a heterotetrameric combination of type I and type II receptors. Type I receptors then activate signal transduction by phosphorylation of Mad (mothers against decapentaplegic). Phosphorylated Mad (pMad) couples with Medea (Smad4) and they then translocate to the nucleus, where they function as DNA-binding regulators of gene expression (Affolter et al., 2001; Keshishian and Kim, 2004; Marques, 2005; Massague and Wotton, 2000).

As for most signaling pathways, activity of the BMP pathway is modulated by conserved extracellular and intracellular regulators. Extracellular BMP antagonists inhibit BMP function by directly binding to the BMP ligand and preventing receptor activation (Chen et al., 2004). In *Drosophila*, *short gastrulation* (*sog*) and *twisted gastrulation* (*tsg*) are BMP antagonists with a

well-characterized function in controlling BMP gradients to shape important developmental events such as dorso-ventral embryonic patterning. (Oda and Akiyama-Oda, 2008). Intracellularly, the conserved inhibitory Smad, Daughters against dpp (Dad), known as Smad 6/7 in vertebrates, competes with Mad for binding to the activated receptor (Inoue et al., 1998). Also, Smurf proteins, such as lethal with a checkpoint kinase (lack), ubiquitinates Mad and targets it for degradation (Liang et al., 2003). At the transcriptional level, Schnurri (Shn) and Brinker (Brk) form transcriptional complexes with Mad and Medea at *cis*-regulatory sequences, often termed BMP-response elements (BRE), to repress or activate transcription in response to BMP signaling (Ross and Hill, 2008).



	Drosophila		Mouse
LIGAND	Glass bottom boat	-	BMP5,6,7,8A,8B
	Decapentaplegic	-	BMP2,4
	Screw	-	BMP5,6,7,8A,8B
TYPE I R	Saxophone	-	ALK-1 ALK-2
	Thickveins	-	ALK-3 ALK-6
TYPE II R	Wishful thinking	-	BMPR-II MISR-II
	Punt	-	ActR-IIA ActR-IIB
R-SMAD	Mad	-	Smad 1 Smad 5 Smad 8
CO-SMAD	Medea	-	Smad 4

Figure 1.2 The BMP pathway in *Drosophila* neurons and their vertebrate orthologs

Components mediating BMP signaling and differentiation in *Drosophila* neurons are underlined. Mouse orthologs of *Drosophila* components are listed on the right had side. (Image made by D.W. Allan).

1.3.1 BMP signaling acts at multiple stages of neuronal development

BMP signaling plays critical roles in the nervous system at multiple stages of development (Liu and Niswander, 2005). During early embryogenesis, BMP signaling mediates a major role in nervous system patterning by regulating the induction of neural tissue from ectoderm. In vertebrates, BMPs are expressed ventrally, while BMP antagonists are expressed dorsally. Reduced BMP signaling due to BMP antagonists in the dorsal ectoderm induces the formation of the neural plate, which will form the central nervous system (Liu and Niswander, 2005). A similar mechanism patterns the *Drosophila* embryo and specifies the region for neuroectoderm formation, but in the reverse orientation. During *Drosophila* embryogenesis, the BMP ligand Decapentaplegic (Dpp) is produced in the dorsal part of the embryo and the BMP antagonist Short gastrulation (Sog) is produced ventrally. Sog antagonizes Dpp ventrally, creating a dorso-ventral gradient of pMad activity. This specifies the formation of the neuroectoderm (future CNS) on the ventral side of the embryo where Sog blocks BMP activity. On the dorsal side, where Sog is absent, Dpp specifies the amnioserosa and the dorsal ectoderm (Ashe, 2005; Ferguson and Anderson, 1992)

A second major role for BMP signaling is in neural tube patterning (Liu and Niswander, 2005; Lupo et al., 2006). During vertebrate neurulation, the neural plate folds, the lateral edges of the neural plate fuse dorsally, and then separate from the non-neural epithelium to form the neural tube. Two distinct organizing centers emerge at this time: the roof plate and floor plate (together with the notochord). The roof plate produces BMPs and the floorplate (and notochord) produce Sonic hedgehog. Both morphogens act in apposing gradients through the neural tube to dorso-ventrally pattern the fate of the neuroepithelial neuronal progenitor cells.

BMP signaling also plays an important role in neuronal specification. In vertebrates, aorta-derived BMPs induce the specification of sympathetic neurons that migrated from the neural crest (Reissmann et al., 1996). In these cells, extrinsic BMP signaling induces the expression of a set of TFs that lead to the specification of noradrenergic sympathetic neurons. BMP-2, -4 and -7 induce the proneural bHLH TF *Mash1*, the paired homeodomain TFs *Phox2a* and *Phox2b*, and the bHLH TF *Hand2*. This combination of TFs then function to differentiate the noradrenergic identity of sympathetic neurons (Sarkar and Howard, 2006; Stanke et al., 2004).

1.3.2 Retrograde BMP signaling in neuronal differentiation

Target-derived retrograde BMP signaling plays conserved roles in neuronal differentiation and synaptic stability in *Drosophila* neurons (Keshishian and Kim, 2004; Marques, 2005) and vertebrate neurons (Ai et al., 1999; Guha et al., 2004; Hall et al., 2002; Hodge et al., 2007; Lee-Hoeflich et al., 2004; Shen et al., 2004; Sun et al., 2007; Withers et al., 2000). Importantly, distinct neurons respond in very unique ways to target-derived BMP signaling. For example, in superior cervical ganglionic neurons, BMP signaling represses PACAP and activates vasoactive intestinal peptide (VIP) (Pavelock et al., 2007). In mouse trigeminal neurons, retrograde BMP-4 signaling regulates TF expression including *Onecut1/2*, *Tbx3* and *Hmx1*. As different subtypes of trigeminal neurons respond differently to BMP signaling, these BMP-dependent responses help differentiate the identity of trigeminal neurons and this helps in the formation of somatosensory facial maps (Hodge et al., 2007). Other TGFβ-family ligands are also involved in inducing target-derived neuronal differentiation. For example the neuropeptide, somatostatin, is induced in ciliary neurons by target-derived activin (Coulombe and Kos, 1997) and CGRP expression in cutaneous afferents is induced by skin-derived activin (Ai et al., 1999).

The manner in which BMP signaling acts at the *cis*-regulatory regions of genes provide a simple rationale for how cell-specific gene regulation is mediated by the BMP pathway. Medea and pMad bind conserved DNA sequences known as the BRE (BMP response element). pMad binds GC-rich sequences, while Medea binds a GTCT sequence. However, both bind DNA with relatively low affinity, and many studies show that they require interaction with other TFs for transcriptional activity (Attisano and Wrana, 2000; Gao and Laughon, 2007; Massague and Wotton, 2000; Shi and Massague, 2003; Wotton and Massague, 2001). Thus, integration of BMP-signaling with cell-specific TF codes would offer a robust means to generate cell-specific gene expression in response to a generic BMP signal.

Our lab was the first to demonstrate that neuronal differentiation in invertebrates can require target-derived signaling (Allan et al., 2003). These studies showed that expression of the neuropeptide gene FMRFa in *Drosophila* Tv4 neurons is dependent upon a target-derived source of the BMP ligand, Gbb, and retrograde trafficking of the BMP signal to the nucleus. We also found that cell-specific combinations of transcription factors intersect with target-derived BMP signaling to specify FMRFa (neuropeptide) expression in Tv neurons (Allan et al., 2005; Miguel-

Aliaga et al., 2004). In this thesis, I describe my identification of an additional three neuropeptides that are dependent upon target-dependent BMP signaling that are selectively expressed in neurons that express the neuropeptide CCAP (CCAP-neurons), showing the common use of target-derived signaling for the differentiation of *Drosophila* neurons.

1.4 PLASTICITY OF NEURONAL GENE EXPRESSION

After the differentiation of subtype-specific neuronal gene expression profiles, neurons can still exhibit remarkable plasticity in gene expression. This enables appropriate responses to physiological or developmental changes as well as injury. While most neurons exhibit some degree of plasticity in gene expression and morphology, the most insightful work has come from studies of neurons that engage in easily assayed phenotypes, such as learning or injury or dramatic functional and/or morphological change related to seasonal changes in neuronal circuitry or metamorphosis in insects (discussed in detail in next section).

A number of well-studied examples serve to underscore the range of plastic changes in which neurons can engage. 1) One well-studied example of plasticity comes from the study of learning and memory in *Aplysia*. Long-term facilitation (LTF) of the sensorimotor synapse can be induced by repeated 5-hydroxytryptamine (5-HT) exposure. LTF at this synapse requires a dramatic increase of expression of the neuropeptide, sensorin, by the sensory neuron (Brunet et al., 1991). This in turn requires a postsynaptic retrograde signal for its induction (Cai et al., 2008; Hu et al., 2006). 2) One other clear example comes from the response of cutaneous sensory neurons to skin wounding. The wound increases local activin and NGF expression in the skin. This then induces expression of the neuropeptide, CGRP, in sensory neurons. This pro-inflammatory neuropeptide then functions to sensitize the local skin area (Cruise et al., 2004; Xu and Hall, 2006). 3) Circuit activity also plays a homeostatic role in neuronal differentiation. Electrical activity in *Xenopus* embryos influences neurotransmitter phenotype in neurons. Decreasing electrical activity by over-expressing the inwardly rectifying K^+ channel (Kir2.1) increased the number of neural tube neurons that express glutamate and the glutamate vesicular transporter (VGluT). Increasing electrical activity by over-expressing voltage-gated Na^+ channels led to a decrease in glutamate and VGluT and an increase in the number of GABAergic and glycinergic neurons (Borodinsky et al., 2004; Flavell and Greenberg, 2008). 4) A clear example of neuronal circuit plasticity in response to seasonal changes comes from the neuronal

circuits that control birdsong. Every year, there is neurogenesis of a new population of neurons that migrate to populate the telencephalic brain regions of male songbirds. These new neurons are incorporated into the motor pathway for song control in order to regulate birdsong during mating season (Wilbrecht and Kirn, 2004).

1.5 METAMORPHOSIS

Most arthropods and amphibians undergo metamorphosis, a process that transforms these animals from a larva into an adult that involves the loss of larva-specific cells and tissues, the adaptation of other cells and tissues to an adult-specific form, and the formation of new adult-specific structures (Paris and Laudet, 2008; Truman, 2005). In *Drosophila*, the larval imaginal discs generate numerous adult body parts such as head structures (eyes, proboscis and antenna) as well as the appendages (wings and legs). Each imaginal disc is initially formed in the embryo as a cluster of 20-40 cells that invaginate from the ectoderm epithelium. Initially, in early larva, each disc is a single epithelial layer, but as the animal nears pupariation, each disc flattens and develops two distinct sides. One side becomes the highly folded disc epithelium while the other side becomes the thin unfolded peripodial membrane. The highly folded disc epithelium will give rise to most of the adult structure. As the imaginal discs develop inside the developing larva, during metamorphosis, the adult structures generated from many of the imaginal discs have to undergo eversion and extension. This occurs during pupal ecdysis at approximately 12hrs after puparium formation (APF) (Bergantinos et al., 2010; Birr et al., 1990; Li and White, 2003). Pupal ecdysis is described in section *1.5.2 Ecdysis*.

The nervous system also undergoes dramatic remodeling during metamorphosis, including gross central nervous system expansion and morphological change, remodeling of existing neuronal morphologies, dramatic neurogenesis, and programmed cell death of larva-specific neurons (Lee et al., 1999; Tissot and Stocker, 2000; Truman, 1990; Winbush and Weeks, 2011). There is a large wave of post-embryonic neurogenesis that occurs prior to and during metamorphosis in brain and thoracic areas of the CNS, as well as in the imaginal discs. This produces sets of mostly interneurons and sensory neurons for adult-specific functions such as vision and olfaction and leg/wing coordination, as well as adding to existing circuits such as the mushroom body which is involved in learning and memory (Tissot and Stocker, 2000). Motoneurons and neurosecretory neurons are largely retained from embryos to be utilized in the

adult, but are morphologically remodeled. Well-studied models of this remodeling include the MN1-5 motoneurons and the FMRFa neurosecretory neurons (Brown et al., 2006; Consoulas et al., 2002). There are also sets of neurons that die during metamorphosis, such as neurons in the abdominal segments of the ventral nerve cord that will not be utilized in the adult (Robinow et al., 1993). A second wave of cell death also occurs post-eclosion, in the adult fly. Examples include CCAP-neurons, which are believed to undergo programmed cell death within 48hrs post-eclosion (emergence as an adult from pupae) (Peabody et al., 2008). One case of trans-differentiation of a neuronal subset has also been uncovered; photoreceptors in Bolwig's organ trans-differentiate by switching rhodopsin gene expression and hence their sensitivity to light from blue/green sensitivity to green-only (Sprecher and Desplan, 2008).

1.5.1 Ecdysteroid cascade

Similar to the changes that occur in vertebrates as they sexually mature through puberty, metamorphosis in *Drosophila* is controlled by a potent hormone cascade. In *Drosophila*, the critical steroid hormone is ecdysone, which is produced and released from the prothoracic gland into the haemolymph. The prothoracic gland is an endocrine gland that is part of a larger structure, the ring gland, which is located immediately anterior to the brain and connected to the brain by tracheal branches and nerves. It is known as the ring gland because in the larva it forms a ring-like structure around the heart. In the haemolymph, ecdysone becomes modified into its active form, 20-hydroxyecdysone (20E). As a lipid steroid, 20E can enter cells, where it binds a nuclear hormone receptor (NHR), the ecdysone receptor (EcR). EcR is bound to its transcriptional heterodimeric partner, Ultraspiracle (Usp), and upon 20E binding, both translocate to the nucleus to modulate gene expression (King-Jones and Thummel, 2005).

Nuclear hormone receptors are a superfamily of TFs comprising six subfamilies in which each NHR contains a conserved DNA-binding domain and most have a ligand-binding domain (LBD). Many bind lipophilic hormones in the cytoplasm facilitating their nuclear translocation, where they regulate transcription of a variety of target genes involved in metabolism, physiology and development. To date, 48 human, 248 *C.elegans* and 18 *Drosophila* NHRs have been identified, many of which are considered orphan receptors as no ligand has been identified. Notably, in *Drosophila*, although fewer NHRs are present, there are still representatives for each of the six NHR subfamilies. The ecdysone receptor (EcR) is one of the best functionally

understood NHRs in *Drosophila* (Galikova et al., 2011; King-Jones and Thummel, 2005; Thummel, 1995).

20E acting via EcR/Usp drives metamorphosis in part through a complex cascade of nuclear hormone receptors. Direct targets of the 20E-EcR-Usp complex include a number of nuclear hormone receptors, *Drosophila* hormone receptor 3 (DHR3; or Hr46), E74, E75, E93, Broad and Ftz transcription factor 1 (Ftz-f1). All of them are transcriptionally regulated by 20E during development (Fig3). However, their differential transcriptional sensitivity to ecdsyone coupled to the cross-regulatory interactions of these nuclear hormone receptors produces a tightly orchestrated cascade that directs the specific phases of metamorphosis (Galikova et al., 2011; King-Jones and Thummel, 2005; Thummel, 1995).

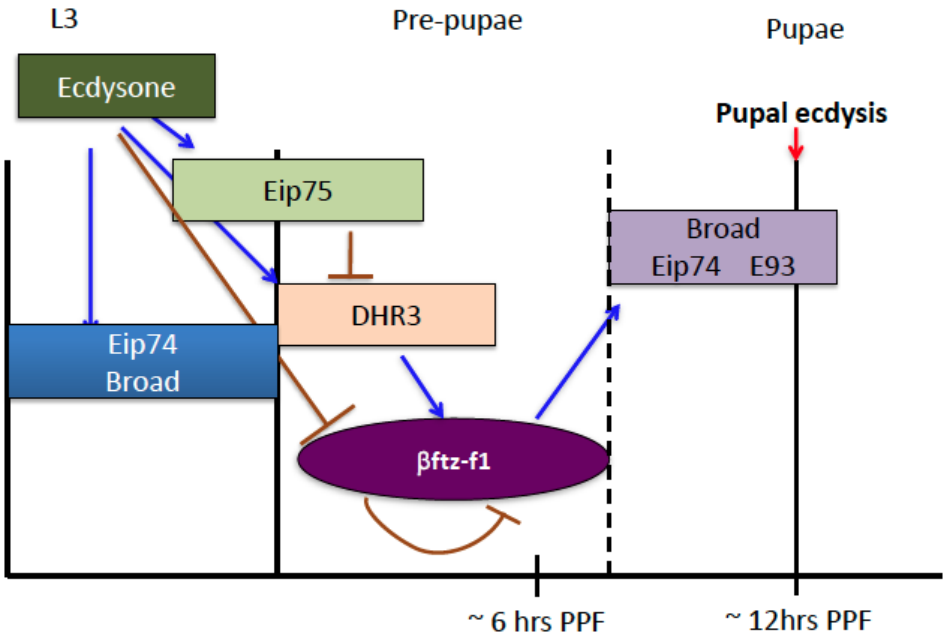


Figure 1.3 The ecdysone-induced nuclear hormone receptor cascade

The ecdysone-induced NHR cascade involves ecdysone-induced expression of nuclear hormone receptors *broad*, *Eip74*, *Eip75* and *DHR3* in neurons. Differential sensitivity of these NHRs to ecdysone, as well as their cross-regulatory interactions ensures that different combinations of NHRs are active at different stages of metamorphosis. (Image made by Lyubov Veverytsa)

1.5.2 Ecdysis

Ecdysis is an essential, innate motor behavior that transitions insects between developmental stages (Ewer, 2005; Truman, 2005). In larvae, larval ecdysis involves shedding the old cuticle between larval stages (Instars). First, the new mouthparts become pigmented, then the new trachea inflates, immediately followed by the animal entering pre-ecdysis. Pre-ecdysis is a period where the animal becomes stationary but exhibits non-peristaltic body wall contractions. This allows air bubbles to be pushed between the old and the new cuticle, to separate the old one away from the animal. This is followed by ecdysis, initiated by lateral head swings and strong body-length peristaltic contractions. These contractions allow the animal to shed the old cuticle (Clark et al., 2004; Park et al., 2002). In pupae, pupal ecdysis involves everting the head and appendages from the interior to the outside of the animal, and then inflating them so that they reach their adult location and size. This is driven by motor activity that drives body wall contractions, which increases the hydrostatic pressure of the haemolymph so that it forces the head and appendages to evert and inflate.

Ecdysis is highly stereotyped and tightly coordinated by a cascade of peptide hormones secreted from a set of dedicated neurons. However, exactly how this cascade mediates all the steps of ecdysis is still controversial (Ewer, 2005; Truman, 2005). In *Drosophila*, falling ecdysone titers trigger a neuropeptide cascade (Fig.4) that acts in a feed-forward manner to direct the ecdysis program (Ewer, 2005). The first known part of the cascade is the secretion of ecdysis triggering hormone (ETH) from peripheral Inka cells, which is first secreted 'spontaneously', perhaps activated by an unknown peptide (Park et al., 2002). *Drosophila* ETH mutants fail to enter the first larval ecdysis and die (Park et al., 2002). ETH stimulates secretion of eclosion hormone (EH) from brain Vm neurons (Horodyski et al., 1993) into the haemolymph. EH, in turn, feeds back to boost ETH secretion, as shown by the reduced ETH secretion observed when EH neurons are genetically ablated (McNabb et al., 1997). Intriguingly, a significant number of EH mutants undergo normal larval ecdysis and show only subtle defects in eclosion (McNabb et al., 1997). This has led to the proposal that there is a redundancy in the cascade. As a result of ETH and EH signaling, CCAP-neurons are activated (Clark et al., 2004). CCAP-neuron ablation in *Drosophila* extends the larval ecdysis program at pre-ecdysis (30% longer) and ecdysis proper

(300% longer), and causes a failure of head eversion and appendage extension during pupal ecdysis, which is lethal (Park et al., 2003). These data show that CCAP-neurons act to facilitate larval ecdysis but are essential for pupal ecdysis. Interestingly, when both EH and CCAP neurons are ablated, the duration of larval ecdysis is significantly lengthened and a large number of larvae die during larval ecdysis, which suggests that CCAP-neurons and EH-neurons act redundantly to direct larval ecdysis (Clark et al., 2004).

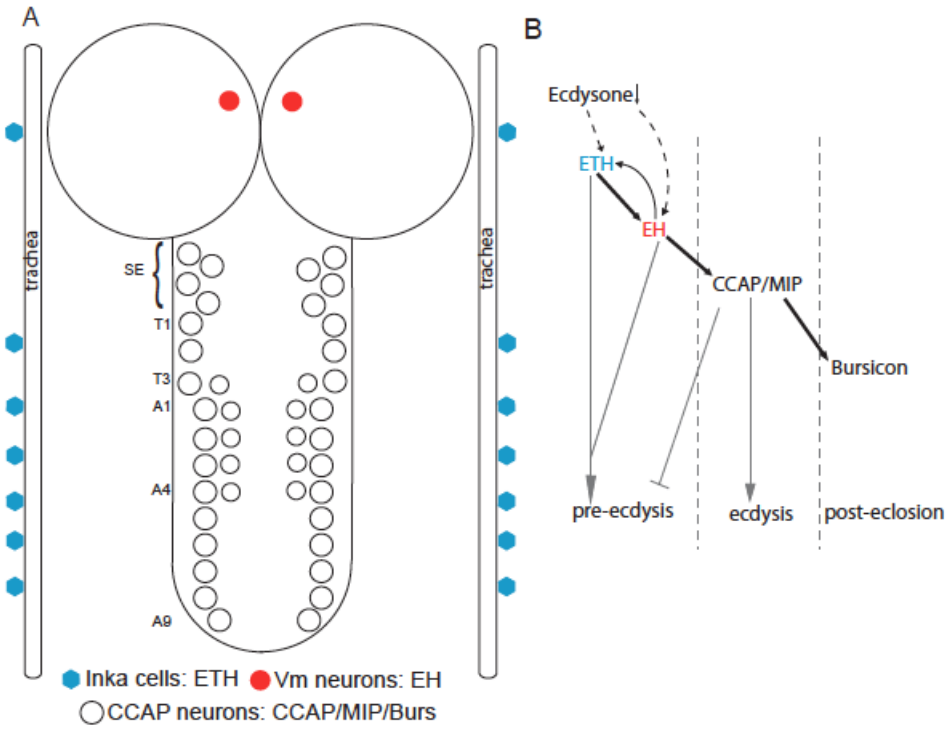


Figure 1.4 Neuropeptide cascade at ecdysis

(A) Location of neurons and the peptide hormones they express during ecdysis. (B) Schematic representation of peptide hormone cascade driving the stages of ecdysis. (Image made by Lyubov Veverytsa)

It is thought that a key role for CCAP-neurons is to be end pre-ecdysis and start ecdysis. In *M.sexta*, CCAP and MIP peptide hormones expressed in CCAP-neurons act synergistically on the isolated/desheathed CNS to terminate ETH-induced fictive pre-ecdysis activity and induce the fictive ecdysis proper motor activity that drives the strong peristaltic waves that persist until the cuticle is shed (Ewer, 2005; Kim et al., 2006a). However, although CCAP alone can induce fictive ecdysis in this preparation, there is no direct evidence for the ability of MIP, on its own, to terminate pre-ecdysis and/or induce ecdysis (Davis et al., 2003; Kim et al., 2006b). In *Drosophila* MIP and CCAP levels decrease in CCAP-neurons (indicative of their secretion) at the time of larval ecdysis (Clark et al., 2004). These data correspond with the sudden increase in MIP and CCAP release during larval ecdysis in *Manduca* and *Bombyx* (Davis et al., 2003). Further experiments assayed CCAP levels in crab (*Carcinus maenas*) and crayfish (*Orconectes limosus*) and found a large increase in circulating CCAP levels during ecdysis (Phlippen et al., 2000). Moreover, intracellular calcium imaging in *Drosophila* CCAP-neurons, in response to exogenous ETH, found that CCAP-neurons were active at the time of pupal ecdysis (Kim et al., 2006b). Thus, it is widely believed that the release of CCAP and MIP is responsible for the behaviors seen during ecdysis (Ewer, 2005; Zitnan et al., 2007). Recently, for the first time, the bursicon hormone, expressed by CCAP-neurons, has also been indirectly implicated in larval ecdysis. Bursicon immunoreactivity decreased in CCAP-neuron type III terminals (on muscle 12) at the time of larval ecdysis, although the role of bursicon in ecdysis is unclear (Loveall and Deitcher, 2010). The model for the role of CCAP-neurons in ecdysis has generally assumed that the same neuronal subsets and the same peptide hormones regulate larval and pupal ecdyses. However, the similarities/differences between larval and pupal ecdysis have not been addressed to date.

1.6 CCAP-NEURONS

The *Drosophila* ventral nerve cord (VNC) has 46 CCAP-neurons (Luan et al., 2006b; Park et al., 2003) comprising two subtypes: CCAP-interneurons (CCAP-IN; *homologs* of *M.sexta* IN704 cells) and CCAP-efferent neurons (CCAP-EN; homologs of *M.sexta* Cells 27) (Fig5). These neurons co-express two neuropeptides (CCAP and MIP) and two peptide hormones (Bursα, Bursβ) (Dewey et al., 2004; Hari et al., 2008; Honegger et al., 2008; Kim et al., 2006b; Luan et al., 2006a; Luo et al., 2005; Vomel and Wegener, 2007; Zhao et al., 2008). CCAP, MIP, Bursα as Bursβ are collectively termed peptide hormones throughout this thesis. In

addition to their role in ecdysis, CCAP-neurons are also critical for key events after eclosion (after emergence of the adult from the pupal case). CCAP-neurons secrete Bursicon-α (Bursα) and Bursicon-β (Bursβ), which act together as the heterodimeric bursicon hormone to inflate the wings as well as harden and tan the cuticle (Dewey et al., 2004; Honegger et al., 2008; Luan et al., 2006a; Luo et al., 2005).

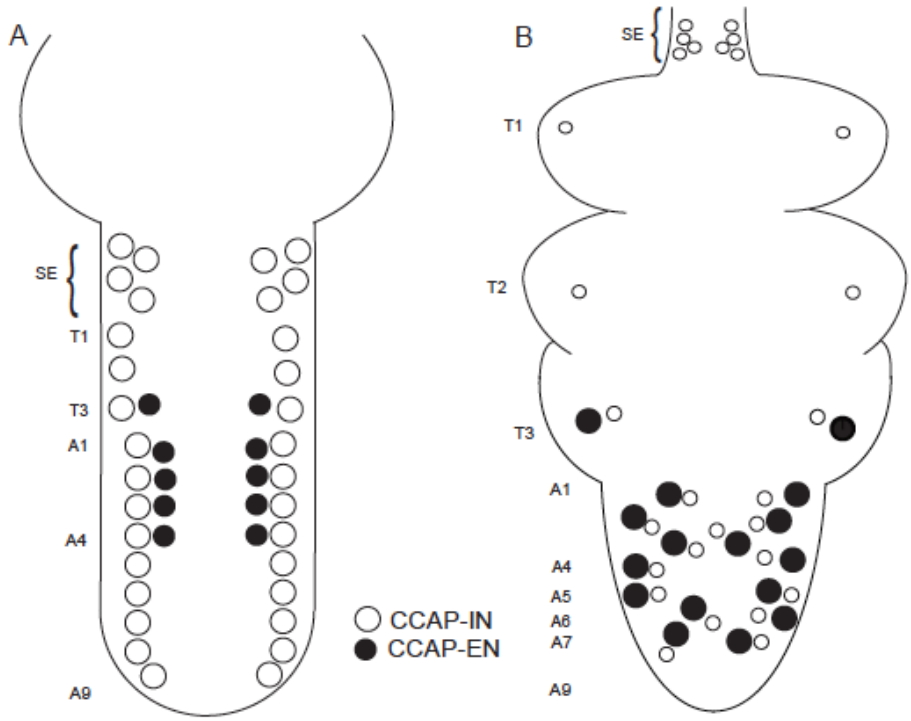


Figure 1.5 Distribution of CCAP-neurons in the *Drosophila* ventral nerve cord

Distribution of *Drosophila* CCAP-neurons in larvae (A) and pharate adult (B). Hollow circles represent CCAP-IN, while solid black circles are CCAP-ENs.
(Image made by Lyubov Veverytsa)

1.6.1 CCAP

Crustacean cardioacceleratoy peptide (CCAP) was originally isolated from the shore crab (*Carcinus maenas*). Experiments using semi-isolated crab heart preparations showed that either native or synthetic CCAP increased heart rate (Stangier et al., 1988). Extensive analysis using an antibody to CCAP allowed identification of its distribution in the central nervous system and its presence in the haemolymph. Using *M.sexta* as a starting point, the *Drosophila melanogaster* CCAP gene was identified and shown to be expressed in a small subset of CNS neurons. Analysis of the results from genetic ablation of *Drosophila* CCAP-neurons further tied CCAP function to both larval and pupal ecdysis (Park et al., 2003; Truman et al., 1996). See section *1.5.2 Ecdysis.*

1.6.2 MIP

The role of MIP is less well understood than that of the other CCAP-neuron peptide hormones. Myoinhibitory peptide (MIP), also known as allatostatin-B, was first identified in *Locusta migratoria*, then subsequently in *Manduca sexta* and other insects including *Drosophila melanogaster*. MIP is co-expressed with CCAP in CCAP-neurons in multiple insects including *Manduca*, *Drosophila*, *Locusta* and *Bombyx* (Blackburn et al., 1995; Lorenz et al., 1995; Lorenz et al., 2000; Schoofs et al., 1991; Williamson et al., 2001). Moreover, experimental evidence has shown that apart from it role in ecdysis (see section ***1.5.2 Ecdysis***), MIP inhibits myogenic contraction of visceral muscles. In *Manduca,* MIPs (Mas-MIPI and Mas-MIPII) have the ability to reduce the rate of ileal peristalsis. In *Periplaneta Americana*, direct application of synthetic MIP peptides inhibits spontaneous activity of the foregut (Blackburn et al., 1995; Predel et al., 2001), as well as suppresses spontaneous contractions of the hindgut and oviduct in *Locusta* (Schoofs et al., 1991).

1.6.3 Bursicon

Bursicon is a heterodimeric hormone composed of two subunits: Bursicon-α (Bursα) and Bursicon-ß (Bursß; also known as partner of bursicon). Bursicon, a 30kD protein, controls cuticle sclerotization in insects (Fraenkel and Hsiao, 1962). It plays a critical role in wing

inflation, cuticle sclerotization and tanning. The nature of Bursicon as a functional heterodimer was not resolved until the discovery of Bursß by Luo et al. in 2005. They demonstrated that Bursicon only activates its GPCR receptor, LGR2, when in the heterodimeric form. Immunohistochemistry to Bursα and Bursß showed that they are co-expressed in CCAP-neurons (Luo et al., 2005).

1.6.4. CCAP neurons are functionally heterogeneous and are remodeled during metamorphosis

CCAP-neurons comprise two primary subsets based on their morphology – efferents and interneurons. The morphology of CCAP-efferents is remodeled during metamorphosis by looking at axonal projections of CCAP-neurons using *CCAP-Gal4, UAS-mCD8GFP*. By 3hrs after puparium formation (APF) (9hrs before pupal ecdysis) pruning starts and is completed by 30hrs APF, although it is unclear how pruned back CCAP-neuronal arbors become by 30hr APF. By 12hrs APF, when pupal ecdysis occurs, CCAP-efferent axons out to the periphery are still extensive, and thus would still be capable of secreting peptide hormones into the haemolymph. After pruning, new adult-specific projections begin to regrow extensively back out to the periphery and within the CNS by 36-42hrs (Zhao et al., 2008).

Another line of evidence for distinct subsets of CCAP-neurons is based on functional analysis. Since the role of Bursicon in cuticle sclerotization and wing inflation offers a very clear phenotypic assay, researchers have used this phenotype to dissect the CCAP-neuronal network in *Drosophila*. During wing inflation in post-eclosion flies, the efferent CCAP-neurons release Bursicon into the haemolymph to mediate changes in the wing cuticle that support expansion and to activate tanning. Also, the subesophageal CCAP-interneurons act within the CNS to regulate the secretion of Bursicon from CCAP-efferent neurons (Luan et al., 2006a; Luan et al., 2006b; Peabody et al., 2008). The Adams group has also studied the activity of CCAP-neuron subsets after ETH exposure at the time of pupal ecdysis, using genetically-encoded Ca^{2+} sensors. They observed increased Ca^{2+} dynamics in Thoracic segment 3 (T3) and Abdominal segments 8/9 (A8/9) CCAP-neurons within 10–15 min, whereas neurons in Abdominal segments A1–4 were activated after approximately 15–25 min delay. These findings led the authors to conclude that differences in activation among CCAP-neuronal subsets lead to different functional roles during the ecdysis sequence. They suggested that the early 10-15min activation related to head eversion during pupal ecdysis and the 15-25min activation was related to leg extension. Together, these

studies provide an indication that CCAP-neurons are a functionally heterogeneous population.

Many questions still remain regarding the differentiation and function of CCAP-neurons. For example, in the many studies of CCAP-neurons, there has been a discrepancy in the number of neurons reported at each developmental stage. This biggest discrepancy is in the number of CCAP-efferent neurons between larvae and adults. The efferent population has 10 neurons in the larva but reports show that there are 16 efferent neurons in pharate adults (Luan et al., 2006a; Peabody et al., 2008). The origin of these extra efferent CCAP-neurons had not been examined. Also, no study has tested the functional heterogeneity of the CCAP-neuronal network with regard to the control of ecdysis. Finally, no study has examined the gene regulatory mechanisms that operate in CCAP-neurons to regulate their differentiation. These questions are addressed in this thesis.

1.7 BROAD RELEVANCE OF STUDYING CCAP-NEURONS

1.7.1 Importance in relation to agriculture and human disease

One reason for the ongoing study of the CCAP-neuronal network is the potential application of understanding this network towards pest control in agriculture and human diseases. Worldwide, insects devastate crops and act as vectors for a wide array of human diseases such as malaria, yellow fever, dengue fever, Chagas disease, Lyme Disease and West Nile Virus. Currently, the most common way of controlling insects is with pesticides. However, as emphasized by the serious environmental impact that arose from widespread use of insecticides such as DDT (dichlorodiphenyltrichloroethane), that were found to be highly toxic to other animals, pesticide use is highly contentious. There has been considerable effort to find other targets for insect control. In this light, identifying the mechanisms of CCAP-neuronal function may be of great utility in the design of targeted insect control methods. Normal CCAP-neuronal function is required for ecdysis and wing inflation in all insects. The ability to prevent insects from either eclosing as adults or of hindering their ability to fly would have tremendous utility in preserving crops and preventing human disease worldwide. Importantly, CCAP-neurons and the peptide hormones CCAP, MIP and bursicon are relatively specific to insects. Thus, pharmaceutical agents could be developed that would disrupt CCAP-neuron function in insects without affecting other animals.

1.7.2 Screening for the basic biological function of neurodegenerative disease genes and ion channels

CCAP-neurons have become a useful model for testing the mechanisms of neurodegenerative diseases, the functional structures of ion channels, and in forward genetic screens to identify the cell-specific expression genes (Bohm et al., 2010; Peabody et al., 2009; Zhao et al., 2008). At the heart of the utility of CCAP-neurons in this regard are two primary factors; i) the dramatic and easily-assayed phenotypes that result from disrupting CCAP-neurons, and ii) the availability of the cell-specific *CCAP-GAL4* driver that can express any *UAS-transgene* selectively in CCAP-neurons.

The mechanisms of FragileX syndrome (FXS) have been analyzed in CCAP-neurons (Gatto and Broadie, 2011). FXS is caused by loss of function of the fragile X mental retardation gene (FMR1). Currently, *Drosophila* pigment dispersing factor (PDF) neurons are also used as an FXS disease model, where recently it was proposed that FXS can be caused by defect in neuronal apoptosis resulting in loss of the ability for neurons to undergo programmed cell death. CCAP-neurons and PDF neurons undergo programmed cell death in adult flies. In dFMR1 mutants, there was a loss of programmed cell death of PDF neurons and a significant delay in apoptosis of CCAP-neurons. Thus, in FXS there is neuronal specificity that underlines new mechanism behind disease process. In principle, any human neurodegenerative disease gene variant can be expressed in CCAP-neurons and tested for its effect on neuronal function. Then, due to the ease of screening a defect in pupal ecdysis and wing inflation, it would be a relatively trivial task to screen for genetic and pharmacological interactors with the human disease gene variant.

CCAP-neurons have also been utilized to test the function of wild-type and modified ion channels on neuronal physiology. Multiple ion channels have been tested in CCAP-neurons to date, including a modified Shaker channel (EKO), a human inward rectifier potassium channel (Kir2.1) and a nonselective TRP cation channel (TRMP8). Expression of these ion channels resulted in range of phenotype from failure of pupal ecdysis to failure to expand wings. These phenotypes indicated that the ion channels were functional as predicted. These studies also proved the utility for the use of those channels to alter the physiology of neurons in a specific way that would be useful to testing neuronal function within a network. Presumably, an

investigator interested in a specific ion channel could screen through multiple protein mutants to identify critical functional domains.

Recently, the CCAP-neuron network was used to test a newly developed Flippase-based intersectional GAL80/GAL4 screening method (known as FINGR) to identify the cell-specific expression of novel enhancer-trapped genes and to allow mapping of neuronal circuits underlying behaviours (Bohm et al., 2010).

As CCAP-neurons become increasingly utilized as a cellular substrate for such studies, it will be increasingly important to understand the mechanisms by which CCAP-neurons function. The studies outlined in this thesis will therefore be of great utility to the research efforts of many research groups.

1.7.3 Model for studying the mechanisms of functional neuronal circuit development and plasticity

CCAP-neurons participate in a complex neuropeptide and peptide hormone cascade that controls an important developmental behavior. In this thesis, we have identified a novel mechanism for neuronal differentiation that is very important in relation to the functional development of neuronal network function. The mechanism of temporally-tuned neuronal differentiation of CCAP-neurons shapes the behavioral output of the developing CCAP-neuronal network. Currently, no examples of this mechanism have been discovered in other organisms, but we believe that once published, other investigators will find that this mechanism is relatively common. One key area that would be worthy of investigation is at puberty in humans and other mammals. Previous studies have implied that changes in neuronal circuitry mediated by steroid signaling are important during puberty. However, the exact mechanism and substrates remain to be discovered (Schulz et al., 2009). Knowledge about neuronal circuitry changes during puberty might be a key to understanding neurological diseases such as autism, schizophrenia and bipolar disease, which typically manifest around the onset of puberty. Thus, by investigating the mechanisms underlying temporally-tuned differentiation in a model such as *Drosophila* CCAP-neurons, we will provide a foundation of understanding similar mechanisms that likely exist in humans and may be an underlying cause for numerous human neurological disorders.

2. RETROGRADE NEURONAL BMP SIGNALING CONTROLS AN ESSENTIAL DEVELOPMENTAL BEHAVIOR IN DROSOPHILA THROUGH NEUROPEPTIDE REGULATION IN A SPECIFIC NEUROSECRETORY SUBSET

2.1 SYNOPSIS

Retrograde BMP-signaling in neurons plays conserved roles in synaptic efficacy and subtype-specific gene expression. However, a role for retrograde BMP-signaling in the behavioral output of neuronal networks has not been established. Insect development proceeds through a series of stages punctuated by ecdysis, a complex patterned behavior coordinated by a dedicated neuronal network. In *Drosophila*, larval ecdysis sheds the old cuticle between larval stages, and pupal ecdysis everts the head and appendages to their adult external position during metamorphosis. Here, we found that mutants of the type II BMP receptor, *wit*, exhibited a defect in the timing of larval ecdysis and in the completion of pupal ecdysis. These phenotypes largely recapitulate those previously observed upon ablation of CCAP-neurons, an integral subset of the ecdysis neuronal network. Here, we establish that retrograde BMP-signaling in only the efferent subset of CCAP-neurons (CCAP-ENs) is required to cell-autonomously upregulate expression of the peptide hormones CCAP, Mip and Bursiconβ. In *wit* mutants, restoration of *wit* exclusively into CCAP-neurons in *wit* mutants significantly rescued peptide hormone expression and ecdysis phenotypes. Moreover, combinatorial restoration of peptide hormone expression in CCAP-neurons in *wit* mutants also significantly rescued *wit* ecdysis phenotypes. Collectively, our data demonstrate a novel role for retrograde BMP-signaling in maintaining the behavioral output of a neuronal network, and uncover the underlying cellular and gene regulatory substrates.

2.2 INTRODUCTION

Retrograde signaling is a conserved mechanism for directing neuronal development and function, acting during the final steps of neuronal development to regulate survival, transmitter phenotype, transcription factor profiles, network connectivity and synaptic efficacy (da Silva and Wang; Hippenmeyer et al., 2004; Ladle et al., 2007; Marques and Zhang, 2006; Zweifel et al., 2005). The BMP pathway has emerged as an important conserved mediator of retrograde signaling. In *Drosophila*, motoneurons and efferent neurosecretory neurons gain access to the BMP ligand, Glass bottom boat (Gbb), from peripheral targets (Allan et al., 2003; McCabe et al., 2003). Gbb activates presynaptic BMP-receptors Wishful thinking (Wit), Thickveins (Tkv) and Saxophone (Sax). Tkv and Sax phosphorylate Mothers against decapentaplegic (Mad) to generate pMad, which translocates to the nucleus to regulate gene expression (Marques, 2005; Shi and Massague, 2003). Retrograde BMP-signaling is a conserved mechanism that directs neuronal terminal differentiation and synaptic efficacy (Allan et al., 2003; da Silva and Wang; Hodge et al., 2007; Marques and Zhang, 2006; McCabe et al., 2003). However, very little is known regarding the function of retrograde BMP-signaling in the behavioral output of neuronal networks.

Drosophila development proceeds through a series of stages that are punctuated by the essential patterned behavior, ecdysis (Thummel, 2001). In larvae, the ecdysis program sheds the old cuticle between each stage. Subsequently, during early metamorphosis, pupal ecdysis everts the head and appendages to the external position of adults (Mesce and Fahrbach, 2002).

Execution of the ecdysis motor program is coordinated by a peptide hormone cascade generated by a dedicated network of neurosecretory neurons and endocrine cells (Ewer, 2005). CCAP-neurons are an essential subset of this network. Their ablation prolongs larval ecdysis and causes a lethal failure of pupal ecdysis (Park et al., 2003). CCAP-neurons co-express the neuropeptides CCAP (crustacean cardioacceleratory peptide) and Mip (myoinhibiting peptide) as well as the two subunits of the bursicon peptide hormone, Bursicon-α (Bursα) and partner of Bursicon (Bursβ). Work in *Manduca* indicates that CCAP and MIP are required for the coordination and execution of ecdysis (Kim et al., 2006a).

We wished to determine whether retrograde BMP-dependent gene expression regulates the behavioral output of neuronal networks. Here, we report that mutants for the BMP type II receptor, *wit*, have a lethal deficit in ecdysis. Our analysis defines the cellular and gene

regulatory substrates that underpin the BMP-dependence of the behavioral output of a neuronal network.

2.3 MATERIALS AND METHODS

2.3.1 Fly stocks

The following fly stocks were used: *dac-GAL4* (Heanue et al., 1999)*; OK6-GAL4*; wit^{A12}*;* wit^{B11} (Aberle et al., 2002); *Ccap-GAL4* (Park et al., 2003); *MHC-*$GAL4^{Geneswitch}$ (Osterwalder et al., 2001); $elav^{GAL4\text{-}C155}$ (Lin and Goodman, 1994); *UAS-*wit^{DN} [*UAS-*$wit^{\Delta I}$*;* intracellular domain deletion (McCabe et al., 2003)]*; UAS-*tkv^{DN} (*UAS-*$tkv^{\Delta GSK}$; GS-box and kinase domain deletion); *UAS-*tkv^{Act}*; UAS-*sax^{Act} (Haerry et al., 1998); *UAS-gbb*; gbb^{1} (Khalsa et al., 1998)*;* Mad^{10}; *Df(2L)JS17* (Mad deficiency) (Sekelsky et al., 1995); *UAS-wit2A* (Marques et al., 2002); *UAS-*$Glued^{DN}$ (*UAS-*$Glued^{s84}$) (Allen et al., 1999); *UAS-nEGFP; UAS-CD8-EGFP* (Bloomington Drosophila Stock Centre). Mutant alleles were kept over *CyO,Act-EGFP* or *TM3,Ser,Act-EGFP* balancer chromosomes. w^{1118} was the control genotype. Flies were maintained on standard cornmeal food (25°C , 70% humidity).

2.3.2 Geneswitch

MHC-$GAL4^{Geneswitch}$ conditionally induces *UAS-transgenes* in the presence of RU486 (Osterwalder et al., 2001). Animals were raised on grape juice-agar plates with yeast paste [untreated or supplemented with 8 μg/ml RU486 (Sigma)]. Controls were raised on both untreated and supplemented yeast paste. Mutants were raised on untreated yeast paste. To restore *gbb* in muscle, animals were raised on supplemented yeast paste.

2.3.3 Generation of UAS-CCAP, UAS-Mip and UAS-Bursβ

Peptide hormone coding sequence (CDS) from pertinent cDNA was amplified by PCR (see Table S1 in the supplementary material). CCAP [EST BO18521; Drosophila Genomics Resource Center (DGRC)] was subcloned into UAS-attB (gift from K. Basler, University of Zürich, Switzerland). Mip (EST GH13904; DGRC) and Bursβ (gift from A. Hsueh, Stanford

University, CA, USA) were subcloned into pUAST. Constructs were injected by Genetic Services (Cambridge, MA, USA). Transformants were confirmed by crossing to *elav*$^{GAL4\text{-}C155}$ and testing immunoreactivity.

2.3.4 Antibodies

Primaries: rabbit anti-CCAP [code 2TB; 1:2000; gift from H. Dircksen (Vomel and Wegener, 2007)]; rabbit anti-Bursα [1:5000; gift from B. White (Luan et al., 2006a)]; mouse anti-Bursβ [1:2000; gift from C. Klein (Luo et al., 2005)]; mouse anti-Mip [antibody 1A4; 1:1000; gift from A. Mizoguchi (Kim et al.; Yamanaka et al.)]; mouse anti-Dac (1:25; clone dac2-3; Developmental Studies Hybridoma Bank); rabbit anti-pMad (1:100; 41D10, Cell Signaling Technology); guinea-pig anti-pMad (1:500; gift from E. Laufer, Columbia University, NY, USA). Secondary antibodies were anti-mouse, anti-guinea-pig, anti-rabbit IgG (H+L) conjugated to DyLight 488, Cy3, Cy5 (1:200; Jackson ImmunoResearch) or Pacific Blue (1:100; Invitrogen/Molecular Probes).

2.3.5 In situ hybridization probes

bursβ DIG-Uracil tagged antisense RNA was generated from a 371bp cloned genomic region (DIG-U-RNA Labeling Kit, Roche). *Ccap, Mip, bursα* DIG-11-dUTP single-stranded DNA probes were generated using primer specific asymmetric PCR (DIG-11-dUTP mix, Roche). For *Mip*, a 923bp region was amplified from EST GH13904 (DGRC). We amplified a 693bp region from cloned genomic *Ccap* and a 484bp genomic from cloned genomic *bursα*. For primers, see Table S1 in the supplementary meterial.

2.3.6 Immunohistochemistry and Multiplex Fluorescent in situ hybridization (FISH)

Standard protocols were utilized, as previously described (Eade and Allan, 2009).

2.3.7 Image analysis

Images were acquired on an Olympus FV1000 confocal microscope and analyzed with Image J (NIH). Image parameters were set to avoid saturation of the brightest immunofluorescence within a data set. To quantify intensity, a mask was made around the CCAP-neuron. Mean pixel intensity was measured within the mask. Background fluorescence of an adjacent area was subtracted. Data presented as mean±s.d for each genotype. These data are presented as the percentage intensity relative to the mean of the control. All images for comparison were identically processed.

2.3.8 Statistics

Statistics were performed using GraphPad Prism 4. Data for immunofluorescence, cell number and larval ecdysis were examined using the D'Agostino and Pearson omnibus normality test. Normally distributed data were compared by two-tailed *t*-test assuming equal variance. Non-normally distributed data were compared with a non-parametric Mann-Whitney test. Data for pupal ecdysis were compared by χ^2 test. Statistical data are presented to the exact *P*-value down to $P<0.0001$. NSD denotes no significant difference at $P>0.05$.

2.3.9 Larval ecdysis

Age-matched embryos were placed on grape juice-agar plates/yeast paste (25°C, 70% humidity). Larvae were recorded though pre-ecdysis and ecdysis proper. Half of the larvae were video recorded (Moticam 2300, 3.0 Mpixel) and analyzed afterwards. The other half were timed visually. Both data sets generated identical results.

2.3.10 Pupal ecdysis

Age-matched embryos were placed in vials containing standard cornmeal food at 25°C, 70% humidity. We measured leg extension in pharate adults using a graticule eyepiece. The thorax/abdomen junction was set as 0 mm. Leg extension posterior of that set point was measured. For each genotype, we determined the percentage of animals within three phenotype

bins: fail (legs extended less than 1 mm), partial (legs extended 1-2 mm) and wild-type (legs fully extended 2-3 mm).

2.4 RESULTS

2.4.1 Pupal ecdysis requires wishful thinking (wit) function in CCAP-neurons

Pupal ecdysis marks the emergence of adult morphology at 12 hours post-puparium formation. It is characterized by eversion and extension of the head and appendages (wings and legs) from their internal position, as imaginal discs, to the exterior. Peptide hormones from a dedicated neuronal network coordinate patterned motor activity that generates muscle contractions to increase hemolymph hydrostatic pressure and force head and appendage eversion and extension (Ewer, 2005; Kim et al., 2006b; Mesce and Fahrbach, 2002).

Null mutants for the BMP type II receptor, *wit*, survive to the pharate adult stage but fail to eclose (Marques et al., 2003). Upon examination of pharate adults in *wit* null mutants (the heteroallelic null combination wit^{A12}/wit^{B11}), in which neuronal BMP signaling is eliminated (Marques et al., 2002), we observed a severe defect in leg and wing extension and a partial defect in head extension that resulted in a `neckless' phenotype. As the most expressive phenotype, we quantified leg extension to evaluate pupal ecdysis (see Materials and methods). In *wit* heterozygotes, leg extension was 90% wild-type, 10% partial and 0% failed (n=30; **Fig. 2.1A,E**). In *wit* mutants, leg extension was 0% wild-type, 35.7% partial and 64.3% failed (n=14; P<0.0001 versus control; Fig. **2.1B,E**).

This *wit* ecdysis phenotype was reminiscent of that reported for CCAP-neuron ablation (*Ccap-KO*): a failure of leg extension and a subtle to severe deficit in head extension (Park et al., 2003). To test whether BMP-signaling in CCAP neurons is essential for pupal ecdysis, we restored *wit* function exclusively in CCAP-neurons in *wit* mutants, using *Ccap-GAL4*. This dramatically rescued the *wit* pupal ecdysis phenotype. Leg extension was rescued to 66.7% wildtype, 23.8% partial and 9.5% failed (n=21 animals; P<0.0001 versus mutant) (**Fig. 2.1C,E**). Moreover, 12% of these animals eclosed as adults (n=11 out of 89 animals), in contrast to 0% of *wit* mutants (n=105), and the rescued adults tanned and inflated their wings (**Fig. 2.1D**). We confirmed that *UAS-wit* did not rescue in the absence of *Ccap-GAL4* (P<0.0001 versus rescued animals) (**Fig. 2.1E**).

BMP signaling in the *Drosophila* nervous system is absolutely dependent upon *wit* (Marques et al., 2002). To confirm that *wit* acts via BMP signaling in pupal ecdysis, we attempted to rescue *wit* mutants with constitutively activated forms of the BMP-specific type I receptors (*UAS-tkv*^{Act}*,UAS-sax*^{Act}). Experiments were performed at 29°C due to the lack of rescue of pMad immunoreactivity in CCAP neuronal nuclei at 25°C, indicating a lack of BMP signaling rescue (see below). At 29°C, pMad immunoreactivity was weakly rescued, although not as strongly as in wild type (see **Fig. S2.1** in the supplementary material). However, this succeeded in significantly rescuing the *wit* mutant phenotype to 19% wild-type, 58% partial and 23% failed leg extension (n=26; P<0.0001 versus mutant), in comparison to *wit* mutants (0% wild-type, 27.6% partial and 72.4% failed; n=29) and to rescue controls in which *Ccap-GAL4* was absent (0% wild-type, 36% partial and 64% failed; n=14) (**Fig. 2.1E**). However, none of these animals eclosed as adults. Although these data confirm that *wit* mediates ecdysis via BMP signaling in CCAP neurons, we suggest two reasons for the partiality of *tkv*^{Act}*/sax*^{Act} rescue. First, constitutive BMP activation in all CCAP neurons caused 80% larval lethality in *wit* mutants. Also, in controls, *Ccap-GAL4*-driven *tkv*^{Act}*/sax*^{Act} resulted in 45% pre-eclosion lethality and 40% failure of wing inflation in adults, indicative of CCAP neuron network dysfunction (Honegger et al., 2008). Second, in surviving *wit* pharate adults, *tkv*^{Act}*/sax*^{Act} only weakly rescued BMP signaling. Together, we suggest that constitutive BMP activation in all CCAP neurons disrupts overall CCAP neuron network function, resulting in significant, yet incomplete, rescue of ecdysis.

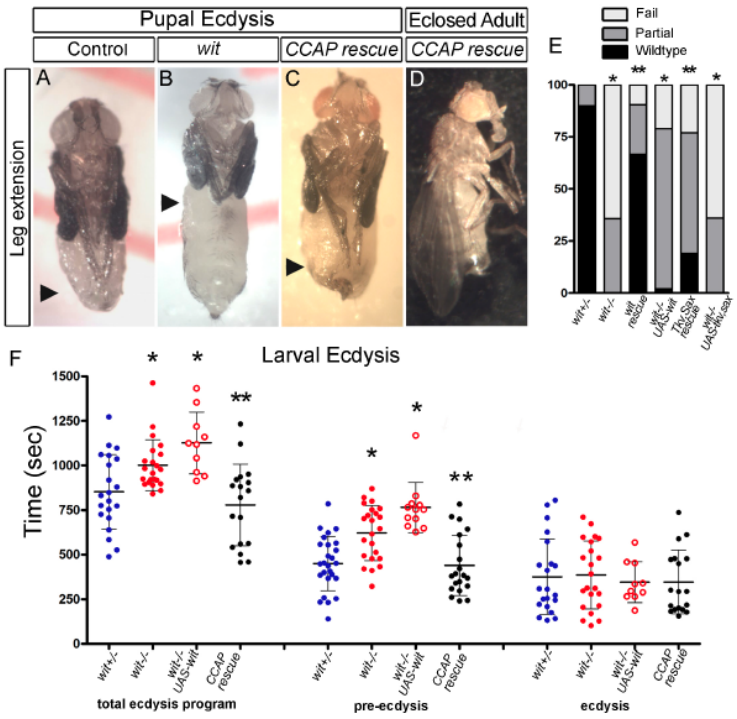


Figure 2.1 *Drosophila* pupal ecdysis requires BMP signaling in CCAP neurons

(**A-D**) Leg extension (limit indicated by arrowheads) in pharate adults of the indicated genotypes (A-C). *wit* mutants exhibit reduced leg extension (B). *UAS-wit* restoration using *Ccap-GAL4* significantly rescued leg extension (C) and eclosion (D). (**E**) Summary of leg extension phenotypes. Both *UAS-wit* and *UAS-tkv^{Act},UAS-sax^{Act}* driven from *Ccap-GAL4* significantly rescued leg extension as compared with mutants and rescue controls (*wit$^{-/-}$ UAS-wit* and *wit$^{-/-}$ UAS-tkv/sax*). Shown is the percentage of animals of each genotype with failed, partial or wild-type leg extension. *, $P<0.0001$ versus control; **, $P<0.0001$ versus mutant and pertinent rescue control. (**F**) Scatter plots depicting the duration of the entire L2/L3 larval ecdysis program, as well as pre-ecdysis and ecdysis proper, for controls (blue), *wit* mutants (red), rescue controls (red circles) and for *UAS-wit* restoration in *wit* mutants using *Ccap-GAL4* (black). The entire ecdysis program and pre-ecdysis were prolonged in *wit* mutants and rescue controls. This was rescued by *wit* restoration in CCAP neurons. Mean ± s.d. *, $P<0.01$ versus control; **, no significant difference (NSD) to control and $P<0.01$ versus mutant. Genotypes: (A) control (*wit^{A12}/+*); (B) *wit* (*wit^{A12}/wit^{B11}*); (C-F) *Ccap* rescue (*Ccap-GAL4,UAS-nEGFP/UAS-wit;wit^{A12}/wit^{B11}*); (E,F) *wit$^{+/-}$* (*Ccap-GAL4,UAS-nEGFP/+;wit^{A12}/+*); *wit$^{-/-}$* (*Ccap-GAL4,UAS-nEGFP/+;wit^{A12}/wit^{B11}*); *wit$^{-/-}$ UAS-wit* (*+/UAS-wit;wit^{A12}/wit^{B11}*); (E) *tkv^{Act}/sax^{Act}* rescue (*Ccap-GAL4,UAS-nEGFP/UAS-tkv^{Act},UAS-sax^{Act};wit^{A12}/wit^{B11}*); *wit$^{-/-}$ UAS-tkv^{Act}/sax^{Act}* (*+/UAS-tkv^{Act},UAS-sax^{Act};wit^{A12}/wit^{B11}*).

2.4.2 Larval ecdysis requires wit function in CCAP-neurons

The larval ecdysis behavioral program is highly stereotyped (Clark et al., 2004). At pre-ecdysis, the animal undergoes compressive body wall contractions that facilitate separation of old and new cuticles. This is followed by ecdysis proper, starting with lateral head swinging and then strong peristaltic waves that shed the old cuticle. *Ccap-KO* animals exhibit prolonged pre-ecdysis (by 30%) and ecdysis proper (by 300%) (Clark et al., 2004).

At the L2/L3 ecdysis, the entire ecdysis program was significantly prolonged in *wit* mutants (**Fig. 2.1F**); 852.1 seconds in controls (n=21 animals) and 1001.6 seconds in *wit* mutants (n=22 animals; P=0.014 versus control). Restoration of *wit* in CCAP-neurons (*Ccap-GAL4*) fully rescued this to 778.7 seconds (n=18 animals; P=0.001 versus mutant; NSD to control). We confirmed that *UAS-wit* did not rescue *wit* mutants in the absence of *Ccap-GAL4*. We examined the contribution of pre-ecdysis and ecdysis proper to the prolonged ecdysis program. Pre-ecdysis took 449.9 seconds in controls (n=26 animals) and was significantly prolonged to 621.7 seconds in mutants (n=23 animals; P=0.0003). Restoration of *wit* in CCAP-neurons fully rescued this to 439.4 seconds (n=20 animals; NSD to control; P=0.0007 versus mutant). In contrast, ecdysis proper was unaffected, with no significant difference between controls, mutants, rescue controls or rescues (**Fig. 2.1F**). Intriguingly, although the timing of ecdysis proper was unaffected in individuals that shed their cuticle, 9/39 (23.1%) of *wit* mutants (at L1/L2 ecdysis) failed to shed their cuticle and died, compared to 1/23 of controls. We conclude that BMP-signaling in CCAP-neurons is necessary for the function of CCAP-neurons in larval pre-ecdysis, and is required for the completion of larval ecdysis in ~25% of animals.

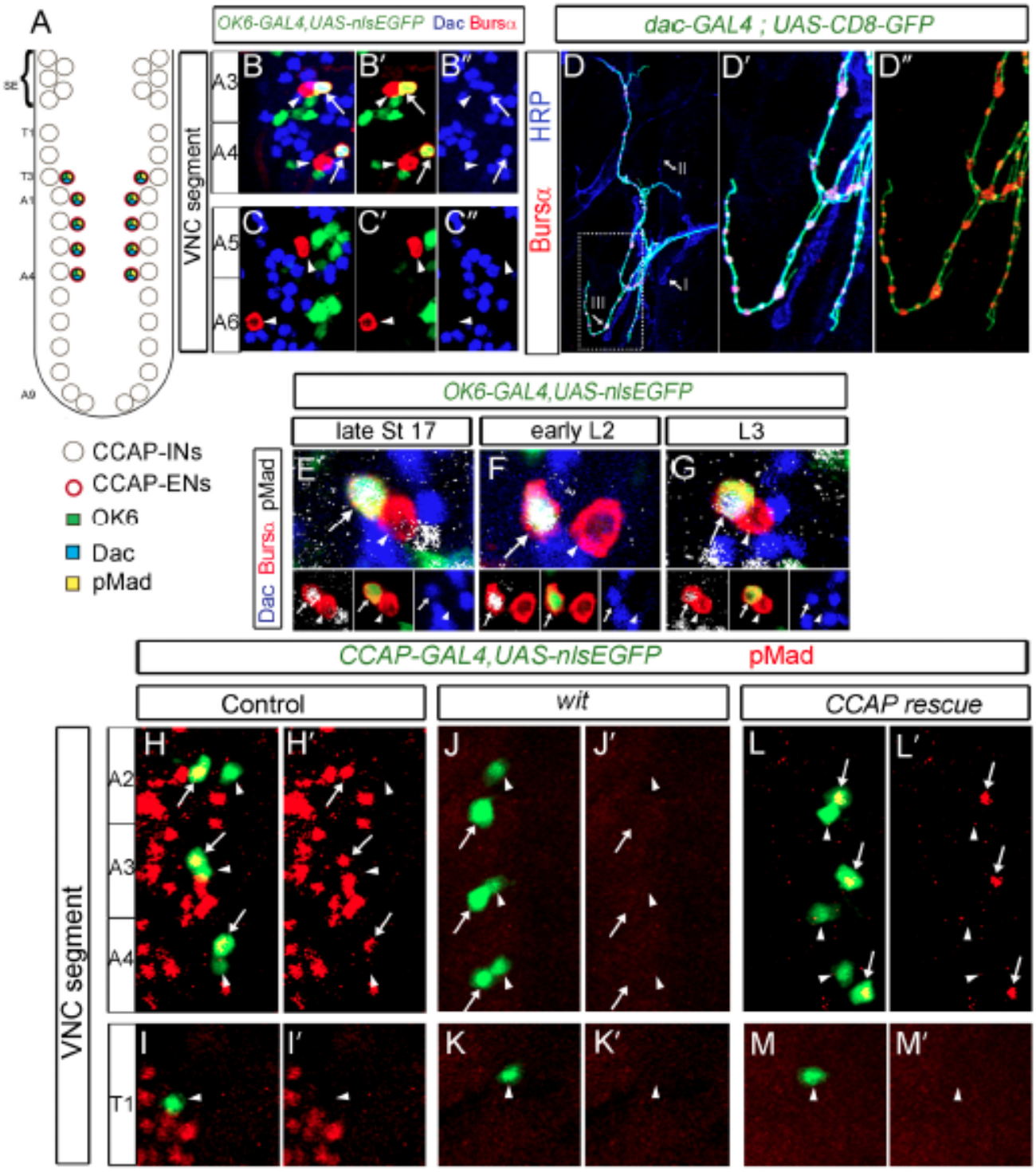
2.4.3 BMP activity in CCAP-neurons is restricted to the efferent subset (CCAP-ENs)

Which CCAP-neuronal subset relies upon BMP signaling for their behavioral output? Previous reports describe 46 CCAP-neurons in the ventral nerve cord (VNC) that almost exclusively express the neuropeptides CCAP and Mip, and the bursicon peptide hormone, a heterodimer of the Bursα and Bursβ subunits (Ewer, 2005; Honegger et al., 2008) (**Fig. 2.2A**). CCAP neurons comprise: (1) an interneuron subset (CCAP-IN), with a single CCAP-IN per hemisegment T1-A9 and five pairs in the subesophageal VNC; and (2) an efferent subset (CCAP-EN), with a single CCAP-EN per hemisegment T3-A4 that projects its axon to terminate

with type III boutons on muscle 12 (Martinez-Padron and Ferrus, 1997; Prokop, 2006; Zhao et al., 2008).

In the *Drosophila* central nervous system, BMP signaling is present in efferent neurons, but absent from interneurons (Allan et al., 2003; Marques et al., 2002). Thus, we postulated that the CCAP-EN subset relays BMP-signaling into ecdysis. This motivated us to identify distinguishing markers for CCAP-ENs and CCAP-INs (**Fig. 2.2E-G**). We examined the expression of numerous transcription factors and enhancer trap reporters commonly used to discriminate neuronal identities in the *Drosophila* nervous system. Of these, *OK6-GAL4* (an enhancer trap expressed in most efferents) and Dachshund [Dac; expressed by efferent neuropeptidergic neurons (Miguel-Aliaga et al., 2004; Miguel-Aliaga et al., 2008)] were found to be expressed in only 10 of the 46 CCAP-neurons. As their location suggested that they were CCAP-ENs, we expressed *UAS-CD8-EGFP* using *OK6-GAL4* or *dac-GAL4* to visualize neuronal morphology. As expected, CD8-EGFP was observed at type III boutons on muscle 12, in hemisegments A1-A5, showing that *OK6-GAL4* and Dac are co-expressed in CCAP-ENs (**Fig. 2.2D**). Notably, although *OK6-GAL4* and Dac are broadly expressed in the VNC, their co-expression can be used to uniquely identify CCAP-ENs (**Fig. 2.2B-C''**).

With these markers, we examined nuclear pMad immunoreactivity, a robust indicator of neuronal BMP-activity (Allan et al., 2003; Marques et al., 2002), in CCAP-neurons. We observed persistent, robust expression of pMad in CCAP-ENs throughout larval and pupal stages, but not in CCAP-INs. (**Fig. 2.2E-I**). In confirmation of previous reports (Marques et al., 2002), pMad immunoreactivity was eliminated in *wit* mutants (**Fig. 2.2J-K'**). As *wit* ecdysis phenotypes were rescued using *Ccap-GAL4* to cell-autonomously restore *wit* **(Fig. 2.1C,D)**, we examined pMad in those animals. Importantly, we found that throughout the entire nervous system, pMad was only rescued in the 10 CCAP-ENs (**Fig. 2.2L,M**). Thus, we conclude that CCAP-ENs relay BMP-signaling into the appropriate execution of ecdysis.


A
OK6-GAL4,UAS-nlsEGFP Dac Bursα
dac-GAL4 ; UAS-CD8-GFP
VNC segment
A3
A4
A5
A6
B
B′
B″
C
C′
C″
D
D′
D″
HRP
Bursα
CCAP-INs
CCAP-ENs
OK6
Dac
pMad
OK6-GAL4,UAS-nlsEGFP
late St 17
early L2
L3
E
F
G
Dac Bursα pMad
CCAP-GAL4,UAS-nlsEGFP pMad
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CCAP rescue
VNC segment
A2
A3
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T1
H
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Figure 2.2 CCAP-ENs exhibit active BMP signaling

(**A**) Distribution of the 36 CCAP-INs and ten CCAP-ENs in subesophageal (SE), thoracic (T) and abdominal (A) segments of the *Drosophila* larval ventral nerve cord (VNC); T3-A4 hemisegments have a CCAP neuron `doublet' comprising one CCAP-EN and one CCAP-IN. (**B-C′**) CCAP-ENs (arrows), but not CCAP-INs (arrowheads), express *OK6-GAL4* and Dac. Larval stage L2 dorsal-half VNC triple labeled (with fluorophore splits) for Bursα (red), *OK6-GAL4* (green) and Dac (blue) in A3-A4 doublets (B-B′) and in A5-A6 CCAP-INs (C-C′). (**D-D′**) Triple label at muscle 12 for *dac-GAL4*;*UAS-CD8-GFP* (green), Bursα (red) and horseradish peroxidase [HRP (blue), which labels all neurons]. Type I, type II and type III bouton types are indicated (arrows). The boxed region in D is magnified in D′,D′, showing Bursα and *dac-GAL4*;*UAS-CD8-GFP* overlap in type III boutons. (**E-G**) Quadruple label for *OK6-GAL4* (green), Dac (blue), pMad (white) and Bursα (red, identifies CCAP neurons), representative of hemisegments T3-A4. Only one of the two CCAP neurons per hemisegment (the CCAP-EN) expresses *OK6-GAL4*, Dac and pMad. Arrows, CCAP-EN; arrowheads, CCAP-IN (**H-M′**) Images from A2-A4 doublets and T1 CCAP-INs showing pMad (red) and *Ccap-GAL4;UAS-nlsEGFP* (green). (**H-I′**) In the control, pMad is present in CCAP-ENs (arrow) but not CCAP-INs (arrowhead). (**J-K′**) In *wit* mutants, pMad immunoreactivity is absent. (**L-M′**) *Ccap-GAL4* restoration of *UAS-wit* rescues pMad immunoreactivity only in CCAP-ENs. Genotypes: (B-C′,E-G) *OK6-GAL4,UAS-nEGFP/+*;(D-D′) *dac-GAL4,UAS-nEGFP/+*;(H-I′) *Ccap-GAL4,UAS-nEGFP/+;wit^{A12}/+*; (J-K′) *Ccap-GAL4,UAS-nEGFP/+;wit^{A12}/wit^{B11}*; (L-M′) *Ccap-GAL4,UAS-nEGFP/UAS-wit;wit^{A12}/wit^{B11}*.

2.4.4 CCAP, Mip and Bursβ expression in CCAP-ENs is BMP-dependent

In *Manduca sexta,* the neuropeptides CCAP and MIP (secreted from CCAP-neuron homologs, Cells 27 and Cells IN704) act to terminate pre-ecdysis and initiate ecdysis proper (Kim et al., 2006a). Studies showing that some, but not all, peptide hormones/neuropeptides are BMP-dependent (Allan et al., 2003; Herrero et al., 2007; Miguel-Aliaga et al., 2008), prompted us to test the hypothesis that peptide hormone/neuropeptide expression in CCAP-ENs is BMP dependent. In controls, we noted that each peptide hormone was expressed in most CCAP-ENs, but was occasionally absent owing to natural variability in expression levels. CCAP and Bursβ expression was downregulated but not eliminated in *wit* mutants. To quantify this, we measured immunofluorescence intensity in every CCAP-EN (see Materials and methods) and present this as a percentage of the mean intensity of controls. Mip expression in *wit* mutants was eliminated in many CCAP-ENs. Intensity measurements proved less reliable for quantifying BMP dependence in such cases, so we quantified the number of CCAP-ENs per VNC that exhibited detectable immunoreactivity. Peptide hormone expression was unaffected in CCAP-INs (see **Table S2.2** in the supplementary material).

In L3 *wit* mutants (wit^{A12}/wit^{B11}), CCAP expression in CCAP-ENs was reduced to 37±26% (n=93 CCAP-ENs) of *wit* heterozygous controls ($wit^{A12}/+$, n=79, $P<0.0001$). Bursβ expression was reduced to 31±19% (n=67) of controls (n=84, $P<0.0001$). This finding was recently independently confirmed by microarray analysis of *wit* mutants, which showed a similar downregulation of Bursβ (Kim and Marques, 2010). Bursα expression was only subtly downregulated in *wit* mutants to 82±28% (n=82) of controls (n=88, $P=0.01$). Mip was eliminated in many CCAP-ENs and severely downregulated in the remainder. In controls, Mip was observed in 7.6±1.1 of the 10 CCAP-ENs per VNC (n=8 VNCs). In *wit* mutants, Mip was weakly expressed in only 1.9±0.9 CCAP-ENs per VNC (n=10, $P<0.0001$). We obtained similar results for each peptide hormone by in situ hybridization (see **Fig. S2.2** in the supplementary material), indicating that BMP signaling is likely to act at the transcriptional level.

Next, as *wit* restoration in CCAP neurons rescued ecdysis and exclusively rescued pMad in CCAP-ENs, we tested whether it also rescues CCAP, Mip and Bursβ (**Fig. 2.3**). In L3 larvae, *wit* restoration fully rescued CCAP immunofluorescence intensity from 25±18% in mutants (n=46 CCAP-ENs) to 91±49% (n=49; $P<0.0001$ versus mutant, NSD to control) (**Fig. 3A-C**). Bursβ immunoreactivity was fully rescued from 50±19% in mutants (n=32) to 85±47% (n=31;

P=0.0002 versus mutant, NSD to control) (**Fig. 2.3I-K**). Mip was partially rescued from expression in only 1.2±0.5 CCAP-ENs per VNC in mutants (*n*=5 VNCs) to 3.4±0.6 (*n*=5; *P*=0.0001 versus mutant, *P*=0.0007 versus control) (**Fig. 2.3E-G**). To further support these results, we co-overexpressed dominant-negative BMP receptors (*UAS-tkv*^{DN}*;UAS-wit*^{DN}) in CCAP neurons to ablate BMP signaling (Eade and Allan, 2009). This reduced CCAP immunofluorescence intensity to 51±39% (*n*=94 CCAP-ENs) of controls (*n*=93; *P*<0.0001) and Bursβ immunofluorescence to 67±38% (*n*=78) of controls (*n*=70; *P*<0.0001). Mip immunoreactivity was largely eliminated by dominant-negative BMP receptor overexpression: Mip was expressed in only 1.9±0.3 CCAP-ENs per VNC (*n*=8), as compared with 5.5±1.1 CCAP-ENs in controls (*n*=10 VNCs; *P*<0.0001). Bursα immunoreactivity was unaffected, remaining at 102±40% (*n*=110 CCAP-ENs) of controls (*n*=110; NSD).

We examined whether BMP signaling acts via the canonical BMP pathway in null *Mad* mutants at early L2 (owing to early lethality). CCAP expression was only observed in 1.8±0.9 CCAP-ENs per VNC in *Mad* mutants (*n*=15 VNCs), as compared with 7.4±2.6 CCAP-ENs in controls (*n*=16; *P*<0.0001) (**Fig. 2.3D**). Similarly, Mip was only expressed in 1.1±0.9 CCAP-ENs per VNC in mutants (*P*<0.0001), compared with 7.8±1.1 CCAP-ENs in controls (**Fig. 2.3H**). Also, Bursβ immunofluorescence intensity declined to 16.6% of controls (*n*=57 CCAP-ENs; *P*<0.0001) (**Fig. 2.3L**). Bursα was only subtly reduced to 71.3% of the control intensity in *Mad* mutants (*n*=141; *P*<0.0001).

Interestingly, while CCAP, Mip and Bursβ expression throughout L3 and pupal ecdysis was robust in most CCAP-ENs, their expression in CCAP-INs at this time was extremely weak (**Fig. 2.3**). We wished to test whether CCAP-INs could also upregulate CCAP, Mip and Bursβ in response to BMP signaling, which would suggest that BMP signaling contributes to a mechanism(s) for differential peptide hormone amplification in CCAP-ENs. We activated BMP signaling in all CCAP-neurons by expressing activated type I receptors *thickveins* and *saxophone* (*UAS-tkv*^{Act}*,UAS-sax*^{Act}) (Allan et al., 2003) using *Ccap-GAL4*. This increased CCAP immunofluorescence intensity in CCAP-INs to 208±19% (n=61 CCAP-INs) of controls (n=72, P<0.0001), but had no effect on the normally robust CCAP-IN expression of Bursα, which was 103±74% (n=69 CCAP-INs) of controls (n=62, P=0.79 NSD). Mip and Bursβ expression is mostly absent in CCAP-INs at L3, however we found that CCAP-INs were capable of increasing peptide hormone expression in response to BMP pathway activation. Quantifying their expression in T3-A8 CCAP-INs (18 CCAP-INs in total), we found that Mip was robustly expressed in 7.9±2.7 T3-A8 CCAP-INs in *tkv*^{Act}*/sax*^{Act} animals (n=12 VNCs), compared to its

normally weak expression in 1.9±1.4 CCAP-INs per VNC in controls (n=12, P<0.0001). Bursβ was robustly expressed in 8.7±1.8 T3-A8 CCAP-INs (n=12) compared to weakly in 2.4±1.4 CCAP-INs per VNC in controls (n=14, P<0.0001).

Collectively, these data suggest that BMP signaling is utilized cell-autonomously to preferentially upregulate peptide hormone expression in CCAP-ENs rather than CCAP-INs.

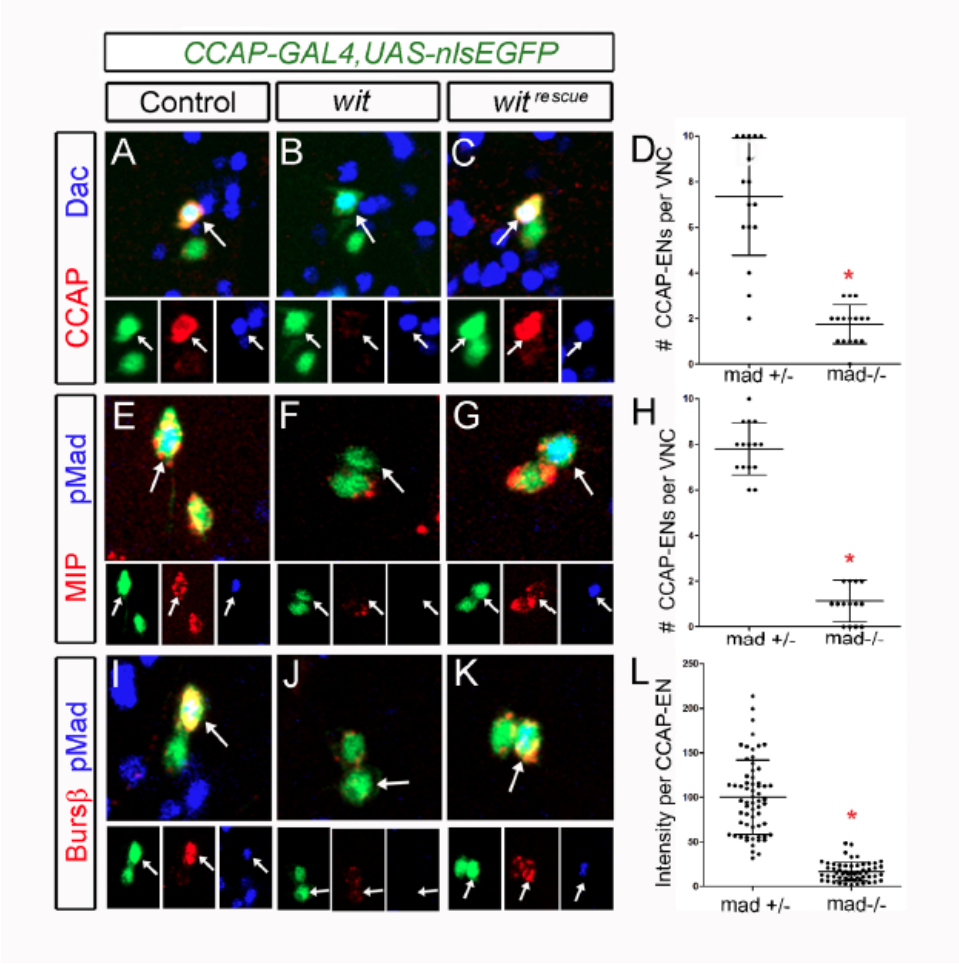

Figure 2.3 Canonical BMP signaling regulates CCAP, Bursβ and Mip in larval CCAP-ENs

(**A-C,E-G,I-K**) Images selected from hemisegments A1-A4. Expression of CCAP (**A-C**), Mip (**E-G**) and Bursβ (**I-K**) at L3. CCAP-ENs (arrows) were identified by location and expression of Dac (blue; **A-C**) or pMad (blue; **E-G,I-K**). (**A,E,I**) Peptide hormone expression in controls. (**B,F,J**) In wit mutants, Mip expression was lost in most CCAP-ENs, whereas CCAP and Bursβ were downregulated. Expression in CCAP-INs was unaffected. (**C,G,K**) Ccap-GAL4 restoration of wit function significantly rescued Mip, CCAP and Bursβ expression in CCAP-ENs. (**D,H,L**) Scatter plots depicting the number of CCAP-ENs per VNC that express CCAP or Mip (**D,H**) or the fluorescence intensity of Bursβ (**L**) for Mad controls and mutants. Mean ± s.d. *, P<0.0001 versus controls. Genotypes: (**A,E,I**) control (*Ccap-GAL4,UAS-nEGFP/+;wit^{A12}/+*); (B,F,J) *wit* (*Ccap-GAL4,UAS-nEGFP/+;wit^{A12}/wit^{B11}*); (C,G,K) Ccap rescue (*Ccap-GAL4,UAS-nEGFP/UAS-wit;wit^{A12}/wit^{B11}*); (D,H,L) *Mad+/–* (*Mad^{10}/+); Mad–/–* (*Df(2R)JS17/Mad^{10}*).

2.4.5 Retrograde Gbb signaling regulates CCAP, Mip and Bursβ in CCAP-ENs.

Considerable evidence indicates that peripheral access to the BMP-ligand Gbb is required for retrograde BMP signaling in efferent neurons (Allan et al., 2003; McCabe et al., 2003). Previous studies implicate the muscle as a primary source of Gbb for motoneurons, and indeed, muscle is known to express Gbb (Ellis et al.; McCabe et al., 2003). We examined whether peripherally acting Gbb triggers retrograde BMP signaling in CCAP-ENs, and tested whether muscle, upon which CCAP-ENs terminate, may act as a potential source. These studies were performed at late L1 owing to the early lethality of *gbb* mutants (**Fig. 2.4**). In controls, CCAP was expressed in 8.6±1.1 CCAP-ENs per VNC (*n*=9 VNCs). In *gbb* mutants, CCAP was only expressed in 1.7±1.2 CCAP-ENs per VNC (*n*=10; *P*<0.0001). We restored *gbb* in muscle using *MHC-GAL4*^{Geneswitch}, which conditionally activates GAL4 activity in muscle after RU486 feeding (see Materials and methods) (Osterwalder et al., 2001). CCAP expression was almost completely rescued by muscle-expressed *gbb* to 7.2±1.7 CCAP-ENs per VNC (*n*=10; *P*<0.0001 versus mutants; *P*=0.05 versus controls) (**Fig. 2.4A**). Mip was expressed in 8.2±1.7 CCAP-ENs per VNC in controls (*n*=16), falling to 2.8±1.4 CCAP-ENs per VNC in mutants (*n*=9; *P*<0.0001). This was significantly rescued by muscle-expressed *gbb*: 4.6±0.7 CCAP-ENs per VNC (*n*=10; *P*=0.001 versus mutants; *P*<0.0001 versus controls) (**Fig. 2.4B**). In *gbb* mutants, Bursβ immunofluorescence intensity fell to 48±17% (*n*=23 CCAP-ENs; *P*<0.0001) relative to controls (*n*=61 CCAP-ENs), and this was significantly rescued by muscle-expressed *gbb* to 69±24% (*n*=37 CCAP-ENs; *P*=0.001 versus mutants, *P*<0.0001 versus controls) (**Fig. 2.4C**).

If a peripheral source of Gbb regulates peptide hormone expression in CCAP-ENs, then retrograde trafficking of the BMP signal to the nucleus would be required, as demonstrated for Tv neurons and motoneurons (Allan et al., 2003; Allen et al., 1999; McCabe et al., 2003). To test this, we blocked retrograde trafficking by expressing dominant-negative Glued (*UAS-Glued*^{DN}) in CCAP neurons. As expected, this eliminated CCAP-EN nuclear pMad immunoreactivity (not shown). Furthermore, it reduced Bursβ from expression in 7.0±1.1 CCAP-ENs per VNC in controls (*n*=10 VNCs) to 1.1±1.6 CCAP-ENs per VNC (*n*=10 VNCs; *P*<0.0001 versus control) and downregulated CCAP intensity to 49±32% (*n*=96 CCAP-ENs) of controls (*n*=10; *P*<0.0001).

It is unclear why muscle-restored Gbb did not fully rescue Mip and Bursβ, especially given the near complete rescue of CCAP. The simplest explanation is the incomplete rescue of BMP signaling itself; pMad immunoreactivity was substantially weaker in muscle-rescued

animals than in controls (see **Fig. S2.1** in the supplementary material) or *wit*-rescued animals (**Fig. 2.2L**). Previous reports also found that muscle-restored Gbb incompletely rescued pMad immunoreactivity and motoneuron neurotransmitter release (McCabe et al., 2003). This was attributed to a partial, but necessary, contribution of Gbb from the central nervous system in addition to that from the muscle. We tested this using *OK6-GAL4* to express Gbb in all efferent neurons in *gbb* mutants, but this failed to rescue any expression of CCAP, Mip or Bursβ in *gbb* mutants (**Fig. 2.4**).

Therefore, we conclude that peptide hormone expression in CCAP-ENs requires peripheral Gbb primarily supplied by the muscle, which establishes a retrogradely trafficked BMP signal to the nucleus. However, it will be interesting to explore whether the incomplete rescue does in fact reflect a necessary contribution from other tissues. Gbb is a secreted protein that is widely expressed, such as by the fat body, somatic and visceral muscle, neurohemal organs and ring gland (Ballard et al., 2009; Doctor et al., 1992; Marques et al., 2003) and may circulate in the hemolymph. Ongoing studies aim to determine whether tissues in addition to muscle are necessary, sufficient or act redundantly to modulate BMP signaling in CCAP-ENs.

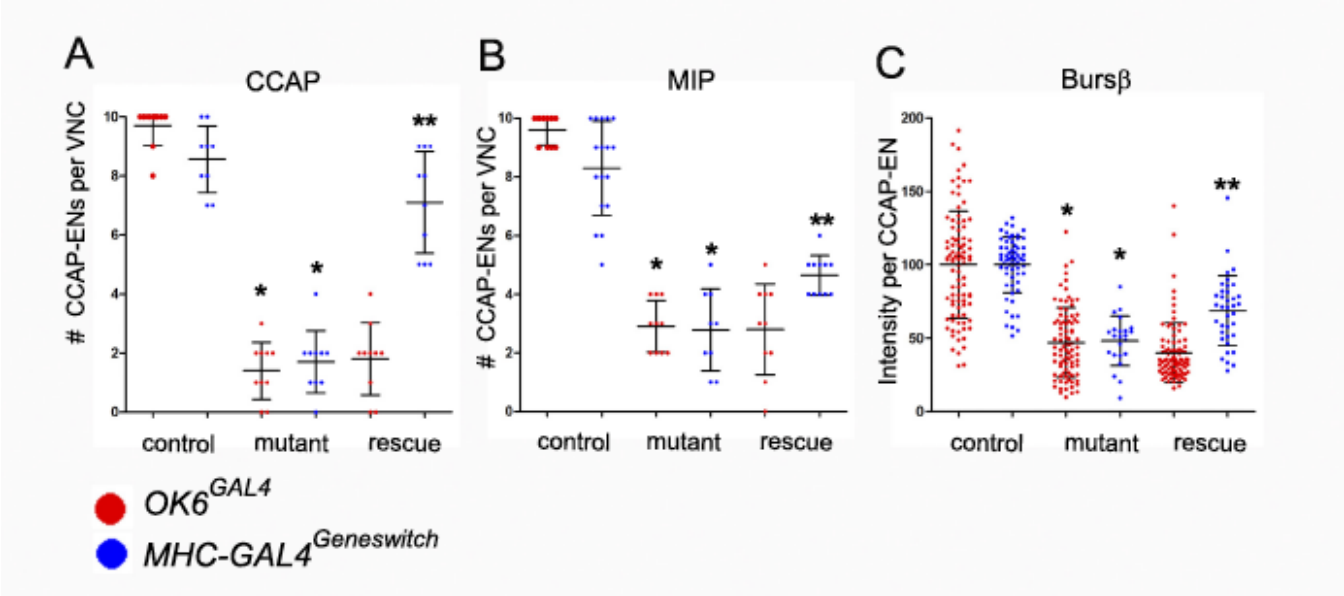

Figure 2.4 Muscle-derived Gbb regulates CCAP, Bursβ and Mip expression

(**A**,**B**) Scatter plots depict the number of CCAP-ENs that express CCAP (A) and Mip (B) per VNC. CCAP and Mip expression was lost in most CCAP-ENs in *gbb* mutants in genetic backgrounds with *OK6-GAL4* (red dots, neuronal GAL4) or *MHC-GAL4*^{Geneswitch} (blue dots, muscle GAL4). When *UAS-gbb* was expressed in *gbb* mutants from *MHC-GAL4*^{Geneswitch}, CCAP and Mip expression was significantly rescued in CCAP-ENs. *UAS-gbb* expressed from *OK6-GAL4* failed to rescue *gbb* mutants. (**C**) Scatter plot of Bursβ intensity in individual CCAP-ENs. Bursβ expression was reduced in CCAP-ENs in *gbb* mutants. When *UAS-gbb* was expressed from *MHC-GAL4*^{Geneswitch}, but not *OK6-GAL4*, Bursβ was significantly rescued in CCAP-ENs. Mean ± s.d. *, $P<0.0001$ versus controls; **, $P<0.0001$ versus mutants. Genotypes: control (*OK6-GAL4,gbb*^{1}*/+;UAS-nEGFP/+*); mutant (*OK6-GAL4,gbb*^{1}*/gbb*^{1}*;UAS-nEGFP/+*); rescue (*OK6-GAL4,gbb*^{1}*/gbb*^{1}*,UAS-gbb;UAS-nEGFP/+*); control (*gbb*^{1}*/+;MHC-GAL4*^{Geneswitch}*/+*); mutant/rescue [*gbb*^{1}*/UAS-gbb,gbb*^{1}*;UAS-gbb/MHC-GAL4*^{Geneswitch} minus RU486 (mutant) or plus RU486 (rescue)].

2.4.6 BMP-signaling regulates pupal ecdysis via peptide hormone expression in CCAP-ENs

As work in *M.sexta* strongly implicated CCAP and MIP in the execution of ecdysis (Kim et al., 2006a), our results led us to the hypothesis that the function of BMP signaling in ecdysis is to uphold functionally competent levels of peptide hormones in CCAP-ENs. We tested this using *Ccap-GAL4* to restore CCAP, Mip and Bursβ expression in *wit* mutants, using *UAS-Ccap, UAS-Mip* and *UAS-burs*β (see Materials and methods).

We repeated control and *wit* mutant experiments in parallel with *UAS*-neuropeptide restoration. In controls, leg extension was 92% wild-type, 8% partial and 0% failed (n=105 animals) (**Fig. 2.5A,D**). In *wit* mutants, leg extension was 4% wild-type, 48% partial and 53% failed (n=105; P<0.0001 versus control) (**Fig. 2.5B,D**). First, we tested restoration of individual peptide hormones using *Ccap-GAL4* in *wit* mutants and present these in order of efficacy: restoration of *UAS-burs*β resulted in 13% wild-type, 54% partial and 33% failed leg extension (n=24; P=0.01 versus mutant); restoration of *UAS-Ccap* resulted in 11% wild-type, 56% partial and 33% failed leg extension (n=9; P=0.003 versus mutant); and restoration of *UAS-Mip* resulted in 9% wild-type, 64% partial and 27% failed leg extension (n=11; P=0.02 versus mutant) (**Fig. 2.5D**). Next, we tested restoration of pairwise combinations of peptide hormones in *wit* mutants (**Fig. 2.5D**). This more dramatically rescued pupal ecdysis phenotypes: leg extension with *UAS-burs*β*/UAS-Mip* was 45% wild-type, 50% partial and 5% failed (n=20; P<0.0001 versus mutant); leg extension with *UAS-burs*β*/UAS-Ccap* was 40% wild-type, 40% partial and 20% failed (n=10; P<0.0001 versus mutant); and leg extension with *UAS-Ccap/UAS-Mip* was 24% wild-type, 64% partial and 12% failed (n=33; P<0.0001 versus mutant). Finally, we tested triple rescue with *UAS-Ccap/UAS-Mip/UAS-burs*β. Leg extension was 20% wild-type, 63% partial and 17% failed (n=24; P<0.0001 versus mutant) (**Fig. 2.5C,D**). However, eclosion was not rescued. These data show that restoring combinations of CCAP, Mip and Bursβ provide significant, albeit incomplete, rescue of the *wit* pupal ecdysis phenotype. Although this indicates that each peptide hormone is necessary for ecdysis, we can only conclude that Bursβ in combination with Mip and/or CCAP is the most important peptide hormone combination. Future analysis of peptide hormone mutants would be required to resolve the relative importance of individual and combined peptide hormones to pupal ecdysis.

Triple rescue was unexpectedly less effective than the double rescues. However, as triple rescue animals were small, relatively immotile and exhibited high larval lethality, we suggest that the increased transgenic load or simultaneous overexpression of all three peptide hormones

in all CCAP neurons reduced individual viability. Also, the rescue of ecdysis was less profound when peptide hormones, rather than *wit*, were restored. We postulate that this might result from interference with CCAP network function due to amplified CCAP/Mip/Bursβ expression in all CCAP neurons, or a reduction in the capacity of *wit* mutant CCAP-ENs to secrete restored peptide hormones. In support of this, we found that type III synapses exhibited a 50% reduction in bouton number and a 35% reduction in branch length (see **Table S2.3** in the supplementary material). Thus, peptide hormone restoration may not fully rescue CCAP neuron function owing to a BMP-dependent deficit in synaptic morphology and function, similar to that seen at type I neuromuscular junctions (Aberle et al., 2002; Marques et al., 2002).

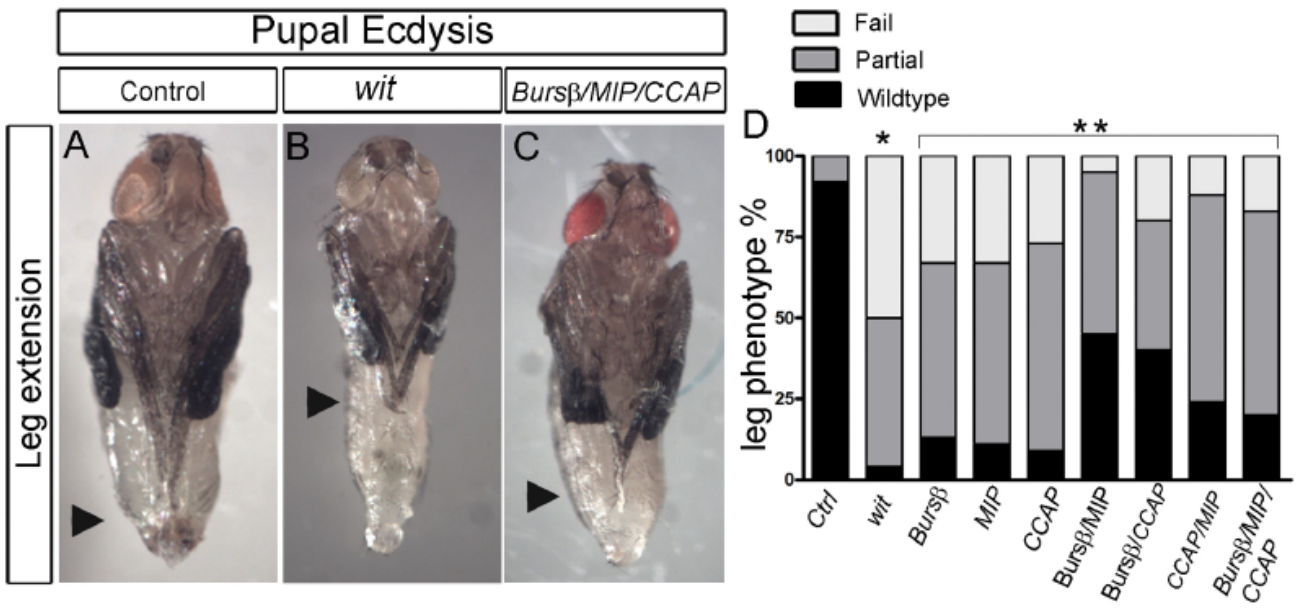

Figure 2.5 Pupal ecdysis is regulated by BMP-dependent peptide hormones in CCAP-ENs

(**A-C**) *Drosophila* pharate adults showing the posterior limit of leg extension (arrowhead). (A) Controls had wild-type leg extension. (B) *wit* mutants had a deficit in leg extension. (C) Triple restoration of *UAS-burs*β, *UAS-Ccap* and *UAS-Mip* in *wit* mutants using *Ccap-GAL4* significantly rescued leg extension. (**D**) Summary of leg extension phenotypes in controls, *wit* mutants, and after *Ccap-GAL4* restoration of *UAS*-peptide hormones in *wit* mutants (either individually or in combination). Shown is the percentage of animals within each genotype that had failed, partial or wild-type leg extension. *, $P<0.0001$ versus control; **, $P=0.01$ versus mutants. Genotypes: (A,D) control (Ctrl) (*Ccap-GAL4,UAS-nEGFP/+;wit*^{A12}*/+*); (B,D) *wit* (*Ccap-GAL4,UAS-nEGFP/+;wit*^{A12}*/wit*^{B11}); (C,D) *burs*β*/Mip/Ccap* (*Ccap-GAL4,UAS-nEGFP/UAS-Ccap,UAS-burs*β*;UAS-Mip,wit*^{A12}*/wit*^{B11}); (D) *burs*β (*Ccap-GAL4,UAS-nEGFP/UAS-burs*β*;wit*^{A12}*/wit*^{B11}); *Mip* (*Ccap-GAL4,UAS-nEGFP/+;UAS-Mip,wit*^{A12}*/wit*^{B11}); *Ccap* (*Ccap-GAL4,UAS-nEGFP/UAS-Ccap;wit*^{A12}*/wit*^{B11}); *burs*β*/Mip* (*Ccap-GAL4,UAS-nEGFP/UAS-burs*β*;UAS-Mip,wit*^{A12}*/wit*^{B11}); *burs*β*/Ccap* (*Ccap-GAL4,UAS-nEGFP/UAS-Ccap,UAS-burs*β*;wit*^{A12}*/wit*^{B11}); *Ccap/Mip* (*Ccap-GAL4,UAS-nEGFP/UAS-Ccap;UAS-Mip,wit*^{A12}*/wit*^{B11}).

2.5 DISCUSSION

We find that retrograde BMP signaling is required to maintain the behavioral output of neuronal networks. Collectively, our data show that retrograde BMP signaling upregulates the expression of a combination of peptide hormones, exclusively in the CCAP-EN subset of CCAP neurons and to a level required for those neurons to contribute to the normal execution of ecdysis behaviors. We discuss our findings in relation to the function of CCAP-ENs in ecdysis, as well as the utility of retrograde signaling as a conserved mechanism for differentiating neuronal identity and regulating behavior.

2.5.1 CCAP-ENs in ecdysis

A feed-forward peptide hormone cascade coordinates ecdysis (Ewer, 2005). Larval and pupal pre-ecdysis is initiated by ecdysis triggering hormone (ETH) from peripheral Inka cells, which stimulates eclosion hormone (EH) secretion from brain Vm neurons. ETH and EH then act together on CCAP-neurons to stimulate CCAP and Mip release. Work on the isolated *Manduca* central nervous system demonstrates that CCAP and MIP synergistically terminate pre-ecdysis and initiate ecdysis proper motor rhythm (Kim et al., 2006a). This is supported by *Drosophila* studies; CCAP neuron ablation prolongs pre-ecdysis and ecdysis proper in larvae, and results in a deficit in the execution of the ecdysis program in pupae that reduces head and appendage eversion and extension (Park et al., 2003). This role for CCAP-neurons has largely been attributed to abdominal CCAP-INs acting locally on motoneurons (Ewer et al., 1997; Gammie and Truman, 1997; Park et al., 2003). However, our observations indicate an essential role for BMP-dependent peptide hormone expression in CCAP-ENs. A detailed analysis of ETH-driven neuronal activity during *Drosophila* pupal ecdysis supports our conclusions (Kim et al., 2006b). This study shows that T3 and A8/A9 CCAP-neurons are active at the start of ecdysis proper, coincident with head eversion, and that A1-A4 CCAP-neurons are active secondarily and throughout the remainder of ecdysis proper, coincident with appendage and head extension. We suggest that the A1-A4 CCAP-neurons active during pupal ecdysis proper and required for leg extension are CCAP-ENs. How would CCAP-ENs that secrete hormones into the haemolymph regulate ecdysis? It has been argued that haemolymph-borne CCAP, MIP and bursicon regulate heart rate, haemolymph pressure and cuticle expansion (Ewer, 2005; Kim et al., 2006b).

However, these peptide hormones may also regulate the activity of central circuits, either indirectly or directly, as established for ETH (Kim et al., 2006b). Genetic analysis of CCAP, Mip and Bursicon peptide hormones and their receptors would provide valuable answers to these questions.

2.5.2 Retrograde BMP-dependent gene regulation in neurons

We find that CCAP-ENs require peripherally-derived Gbb for BMP signaling and enhanced peptide hormone expression. CCAP-EN axons terminate on muscle 12. Muscle expresses Gbb (Ellis et al.; McCabe et al., 2003) and we find that muscle-derived (but not neuronal-derived) Gbb significantly rescued BMP signaling and peptide hormone expression in CCAP-ENs. We also observe pMad immunoreactivity and GFP-Thickveins (expressed from *Ccap-GAL4*) within type III boutons (not shown), indicative of local BMP signaling (O'Connor-Giles et al., 2008). Thus, together with reports that muscle-derived Gbb is sufficient for retrograde BMP signaling in motoneurons (McCabe et al., 2003), the weight of evidence supports the somatic muscle as a primary target for Gbb access for CCAP-ENs. However, we do not rule out the possibility that other sources for Gbb exist, perhaps secreting the ligand into the circulating haemolymph. In this regard, it is notable that Ballard et al (2009) reported that, in *gbb* mutants, restoration of Gbb in another peripheral tissue, the fat body, failed to rescue BMP-signaling in neurons, suggesting that distant signaling via the haemolymph is not sufficient. Further detailed analysis will be required to identify necessary and/or redundant roles for other tissues in neuronal BMP-signaling.

Although muscle is the likeliest target with respect to *gbb*, the muscle is unlikely the primary target for CCAP-EN peptide hormones. Ultrastructural analysis shows that Type III boutons lie superficially on the muscle surface and that dense core vesicles exocytose toward the haemolymph and muscle (Atwood et al., 1993; Prokop, 1999, 2006). Furthermore, bursicon immunoreactivity is detectable in the haemolymph (Luan et al., 2006a). CCAP-EN peptide hormones are known to target the wing, cuticle, and cardiac and visceral muscle, but not the somatic muscle (Ewer, 2005; Honegger et al., 2008). This situation is unusual, as target-derived factors are typically viewed as influencing neuronal gene expression profiles pertinent to the target itself (da Silva and Wang). Footpad-derived cytokines induce cholinergic differentiation of sympathetic neurons required for footpad sweat secretion (Francis and Landis, 1999). Axial differences in BMP4 ligand expression in the murine face direct subset-specific gene expression

in innervating trigeminal neurons that shapes the formation of somatosensory maps (Hodge et al., 2007). Activin and nerve growth factor in the developing skin induce expression of the hyperalgesic neuropeptide calcitonin gene-related peptide (CGRP) in nociceptive afferents (Hall et al., 2002; Patel et al., 2000).

Without evidence for such a mutualistic relationship, what purpose could retrograde BMP-dependent gene expression play in CCAP-ENs? The tremendous cellular diversity of the nervous system is achieved through the progressive refinement of transcriptional cascades within increasingly diversified neuronal progenitor populations (di Sanguinetto et al., 2008; Guillemot, 2007; Skeath and Thor, 2003). Subsequently, retrograde signaling further differentiates the expression profile in postmitotic neurons (Hippenmeyer et al., 2004; Ladle et al., 2007; Nishi, 2003). In such cases, unique access to extrinsic ligands allows for a certain mechanistic economy, enabling a somewhat common regulatory landscape to be adapted towards distinct gene expression profiles. In this context, we postulate that retrograde BMP-signaling functions to diversify the expression levels of peptide hormones in CCAP-neurons. *Drosophila* interneurons and efferents can be sharply distinguished on the basis of BMP activity (Allan et al., 2003; McCabe et al., 2003). Moreover, we show that BMP activation in CCAP-INs is capable of enhancing their peptide hormone expression, implicating a similar gene regulatory landscape in CCAP-ENs and CCAP-INs. Thus, the BMP-dependence of CCAP, Mip and Bursβ offers a simple solution to the problem of how to selectively enhance peptide hormone expression in CCAP-ENs.

BMP-signaling offers an additional advantage to neuronal diversification. Studies of axial patterning in *Drosophila* have unveiled a wealth of mechanisms that diversify and gauge transcriptional responses to BMP-signaling (Raftery and Sutherland, 2003; Ross and Hill, 2008). These mechanisms revolve around the outcome of pMad/Medea activity at a gene's cis-regulatory sequence, as influenced by their affinity for specific cis-regulatory sequences and local interactions with other transcription factors, co-activators and co-repressors. As a result, pMad/Medea activity can be extensively shaped to generate gene- and cell-specific responses and determine whether genes are on or off or up- or downregulated. This flexibility is likely to underpin the differential sensitivity of CCAP, Mip and Bursβ to a common retrograde BMP signal within a single cell, as well as the utility of BMP signaling as a common retrograde regulator of subset-specific gene expression in distinct neuronal populations (da Silva and Wang).

Finally, the differential regulation of Bursα and Bursβ is intriguing because they are believed to only function as a heterodimer (Honegger et al., 2008). While we do not discount the possibility of functional homodimers, we postulate that the selective BMP-dependence of Bursβ may be an efficient mechanism for modulating the activity of the active bursicon hormone. This would be analogous, and perhaps orthologous, to the regulation of follicle-stimulating hormone in mammals. Its cyclical upregulation during the oestrous cycle is dictated by regulation of only one of its subunits, FSHβ, by the TGFβ family ligand, activin (Gregory and Kaiser, 2004; Jorgensen et al., 2004).

2.5.3 Retrograde BMP-signaling in behavior

Numerous studies have described the impact of retrograde signaling on neuronal network formation and function. During spinal sensory-motor circuit development, retrograde neurotrophin signaling induces specific transcription factor expression in motoneurons and Ia afferents that is required for appropriate motor-sensory central connectivity that, when inoperative, results in ataxic limb movement (Arber et al., 2000; Ladle et al., 2007). Similarly, murine trigeminal neurons utilize spatially-patterned BMP4 expression in the developing face to target their centrally-projecting axons in a somatotopically-appropriate manner (Hodge et al., 2007). Retrograde signaling also modulates physiologically-responsive neuronal gene expression. In vertebrates, skin injury induces cutaneous activin and nerve growth factor expression, which retrogradely upregulates sensory neuron expression of CGRP, which mediates hyperalgesia (Xu and Hall, 2006, 2007). In sensory-motor circuits of Aplysia, retrograde signals are required to upregulate presynaptic sensorin, a neuropeptide required for long-term facilitation of the sensorimotor synapse (Cai et al., 2008).

Our evidence suggests that the function of BMP-signaling is not mediated within a specific developmental window, but is required on an ongoing basis. The *Ccap-GAL4* transgene is not active until late larval stage L1, after CCAP-neuron network assembly and peptide hormone initiation. Yet, *wit* phenotypes were significantly rescued using *Ccap-GAL4.* Together with our observation of persistent pMad immunoreactivity in CCAP-ENs, we conclude that BMP-signaling acts permissively to maintain the capacity of CCAP-ENs to contribute to ecdysis, rather than acting phasically at ecdysis to instructively activate ecdysis behaviors or enable CCAP-ENs to contribute. Such a maintenance role is supported by our previous work showing

that maintained expression of the neuropeptide FMRFa requires persistent retrograde BMP-signaling (Eade and Allan, 2009). We also found type III synapses on muscle 12 have significantly fewer boutons and shorter branches in *wit* mutants, implicating a role for BMP signaling in CCAP-EN synaptic morphology, as first described for type I neuromuscular junctions in *wit* mutants (Aberle et al., 2002; Marques et al., 2002). It will be of interest to investigate whether dense core vesicle exocytosis is also perturbed in *wit* mutants, akin to the reduced synaptic vesicle exocytosis at type I boutons in *wit* mutants (Aberle et al., 2002; Marques et al., 2002).

3. TEMPORALLY-TUNED DIFFERENTIATION OF A DROSOPHILA CCAP-NEURON SUBSET REMODELS A FUNCTIONING NETWORK TO SUPPORT A BEHAVIORAL SWITCH

3.1 SYNOPSIS

During insect metamorphosis, larval neuronal networks undergo extensive remodeling by recruiting newborn neurons from post-embryonic lineages and restructuring connectivity. CCAP neurons and the peptide hormones they secrete regulate ecdysis, an essential behavior that sheds the old cuticle between larval stages, and in pupae externalizes the head and appendages that had developed internally. Ablation of all *Drosophila* CCAP-neurons was shown to extend the duration of larval ecdysis and cause a lethal failure of pupal ecdysis. In this study, we show that the larval CCAP-neuron network is remodeled prior to pupal ecdysis by recruitment of twelve 'late' CCAP-neurons. Unexpectedly, these neurons were not recruited from post-embryonic lineages. Instead, we found that late CCAP-neurons become postmitotic in the embryo but do not to terminally differentiate into peptide hormone-expressing CCAP-neurons until pupariation. Moreover, we found that late CCAP-neurons were sufficient for wildtype pupal ecdysis, after the targeted ablation of all other 46 'early' CCAP-neurons. Further analysis indicated that ecdysone receptors EcR-A and EcR-B1 as well as the nuclear receptor, ftz-f1, act as a differentiation trigger for the late CCAP-neurons. These data demonstrate that temporally-tuned differentiation of specific neuronal subsets provides a hard-wired developmental mechanism to functionally remodel a neuronal network.

3.2 INTRODUCTION

Peptidergic neurosecretory cells play critical roles in animal behavior (Hokfelt et al., 2000; Nassel and Winther, 2010). Insect ecdysis is an essential developmental behavior that is orchestrated by a peptide hormone cascade produced by a dedicated network of neurosecretory neurons and endocrine cells (Ewer, 2005). The ecdysis behavioral sequence is reiterated at the transition of major developmental stages; shedding the old cuticle between larval stages (larval ecdysis), everting the head and appendages to their external adult position in 12hr pupae (pupal

ecdysis), and eclosion from the pupal case (adult ecdysis) (Mesce and Fahrbach, 2002). Previous work demonstrated the importance of the CCAP-neuron population to ecdysis; their targeted ablation in *Drosophila* slows larval ecdysis but causes a lethal failure of pupal ecdysis (Park et al., 2003). However, although the behavioral sequence of larval and pupal ecdysis are distinct, the underlying neuronal network is assumed to be the same (Ewer, 2005).

Neuronal networks can be remodeled to match network output to the physiological and behavioral demands of the organism (Brunet et al., 1991; Cruise et al., 2004; Wilbrecht and Kirn, 2004). Insect metamorphosis provides particularly dramatic examples of network remodeling. Most larval neurons undergo morphological restructuring to rewire into adult networks. Post-embryonic waves of neurogenesis in larvae and pupae also add newborn neurons to existing or adult-specific networks. Also, programmed cell death removes certain larval-specific neurons (Brown and Truman, 2009; Lee et al., 1999; Levine et al., 1995; Winbush and Weeks, 2011; Zheng et al., 2003). Previous work had shown that CCAP-neurons undergo morphological remodeling through metamorphosis (Zhao et al., 2008). In this study, we now show that 12 additional 'late' CCAP-neurons are added to the larval CCAP-neuron population at pupariation that are entirely sufficient for pupal ecdysis. Further analysis showed that late CCAP-neurons are born in the embryo in segments A5-A9, but unlike the other CCAP-neurons, their terminal differentiation requires ecdysone signaling and the nuclear hormone receptor ftz-f1 at pupariation. These data demonstrate that the timing of neuronal subtype differentiation can vary in a segment-specific manner in the *Drosophila* ventral nerve cord and highlight temporally-tuned terminal differentiation as a mechanism to support the functional remodeling of a neuronal network.

3.3 MATERIALS AND METHODS

3.3.1 Fly stocks

dac^{GAL4} (Heanue et al., 1999); *CCAP-GAL4* (Park et al., 2003); *OK6-GAL4* (Aberle et al., 2002); *$tubP\text{-}GAL80^{TS}$*(McGuire et al., 2003); *UAS-nEGFP; UAS-CD8-GFP; $UAS\text{-}EcR\text{-}A^{DN}$; $UAS\text{-}EcR\text{-}B1^{DN}$; $UAS\text{-}EcR\text{-}B2^{DN}$* (Bloomington *Drosophila* Stock Center)*; Act-FRT.STOP.FRTnlacZ, UAS-Flp* (Struhl and Basler, 1993); *hs-ftz-f1* (Woodard et al., 1994). Lethal alleles maintained over *CyO,Act-EGFP* or *TM3,Ser,Act-EGFP* balancer chromosomes. *w^{1118}* was the control genotype. Flies were maintained on standard cornmeal food (25°C, 70% humidity).

3.3.2 Immunohistochemistry

Standard immunohistochemical protocols were utilized, as previously described (Eade and Allan, 2009). Primary antibodies: rabbit anti-CCAP [code 2TB; 1:2000; gift from H. Dircksen (Vomel and Wegener, 2007)]; rabbit anti-Bursα [1:5000; gift from B. White (Luan et al., 2006a)]; mouse anti-Bursβ [1:2000; gift from C. Klein (Luo et al., 2005)]; mouse anti-Dac (1:25; clone dac2-3; Developmental Studies Hybridoma Bank); rabbit anti-pMad (1:100; 41D10, Cell Signaling Technology); chicken anti- ßgal (1:1000, ab9361, Abcam). Secondary antibodies: Anti-mouse, anti-chicken, anti-rabbit IgG (H+L) conjugated to DyLight 488, Cy3 and Cy5 (1:200; Jackson ImmunoResearch).

3.3.3 Image and statistical analysis

All analyses was performed as described (Veverytsa and Allan, 2011).

3.3.4 Heat shock-ftz-f1 induction

Larvae were heat shocked at 37ºC for 1 hr, then allowed to recover at 25ºC for 2-4 hrs before dissection and analysis.

3.4 RESULTS

Drosophila larvae have 46 CCAP-neurons that we term 'early' CCAP-neurons, due to their early larval differentiation. Amongst these, 10 CCAP-ENs are distributed bilaterally in ventral nerve cord (VNC) segments T3-A4. CCAP-ENs co-express the peptide hormones CCAP, MIP, Bursα and Bursβ and can be identified by co-expression of the enhancer trap *OK6-GAL4*, the transcriptional regulator Dachshund (Dac) and nuclear accumulation of phosphorylated Mad (pMad), indicative of active BMP signaling (**Fig 3.1 *A-B***) (Allan et al., 2003; Marques et al., 2002). In contrast, numerous reports indicate that pharate adults (immediately prior to eclosion) have 16 CCAP-ENs that co-express Bursα, Bursβ, CCAP and *CCAP-GAL4* (Luan et al., 2006a;

Peabody et al., 2008; Zhao et al., 2008). Here, we wished to identify CCAP-neuronal subsets in pharates. Bursα and CCAP immunoreactivity were observed in 16 large dorsal CCAP-neurons in T3-A7. These were CCAP-ENs based on their size and co-expression of *OK6-GAL4*, Dac and pMad (Zhao et al., 2008) (**Fig 3.1 *C,C',D***). CCAP-INs were smaller and expressed CCAP and *CCAP-GAL4*, but not Bursα, *OK6-GAL4*, Dac or pMad (**Fig 3.1 *C,D,* Fig 3.4 *A,B*, Fig 3S5 *B***). We also identified six previously uncharacterized posterior lateral CCAP-neurons (CCAP-PL), located in segments A8 and A9, that express CCAP, *CCAP-GAL4*, *OK6-GAL4* and pMad, but not Dac, Bursα or Bursβ (**Fig. 3.1 *C', D*, Fig. 3S1 *A-C***). We refer to the late-emerging populations of neurons as 'late' CCAP-neurons.

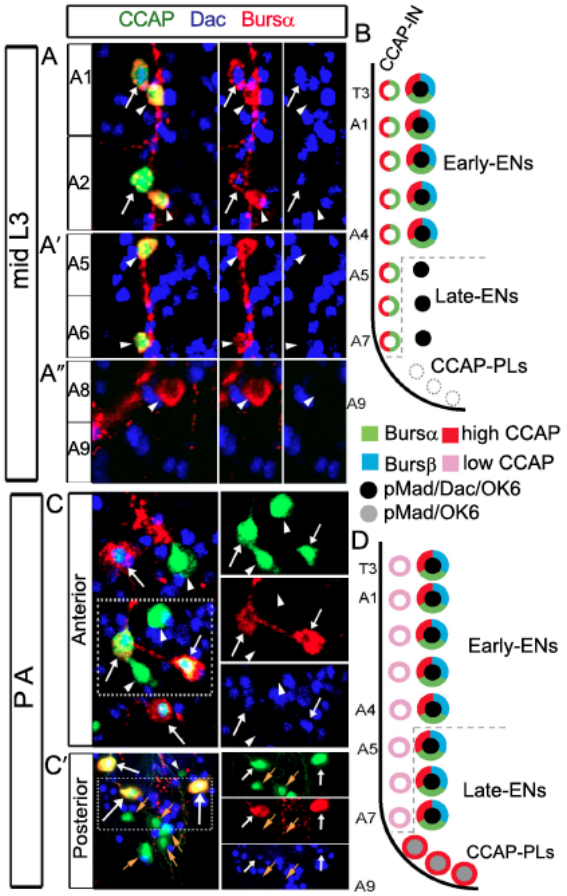

Figure 3.1. The CCAP neuron population expands between larvae and pharate adults

Pharate adults (PA) have additional CCAP-neurons compared to larvae. (**A-A'', C-C'**) Triple label and fluorophore splits of *CCAP-GAL4,UAS-nEGFP* (green), anti-Dac (blue) and anti-Bursα (red) in mid L3 hemisegments A1-A9 (A-A'') and PA abdominal segments (C-C'). CCAP-ENs distinguished by Dac^{+ve} expression. (**B,D**) Depiction of gene expression in all T3-A9 VNC CCAP-neuron subsets at mid L3 (B), and PA (D). Dotted line delimits early and late subsets of CCAP-neurons. (**A-B**) At mid L3, early CCAP-ENs (arrow) and CCAP-INs (arrowhead) form 'doublet' in T3-A4 hemisegments. In A5-A9, only Dac^{-ve} CCAP-INs are seen. (**C,C'**) Triple label and fluorophore splits of boxed regions. At PA, 16 CCAP-ENs (white arrows; Dac^{+ve}, Bursα^{+ve}) are seen in the anterior (T3-A4) and posterior (A5-A9) abdominal VNC. CCAP-PLs are also seen in A8-A9 segments (orange arrows; Dac^{-ve}, Bursα^{-ve}). CCAP-PLs distinguished by position, expression of CCAP and pMad and absence of Dac and Bursα (see text and **Fig 3S1 A-C** for details). **Genotype.** *CCAP-GAL4,UAS-nEGFP/+*.

3.4.1 Late CCAP-neurons are sufficient for pupal ecdysis

Previous studies found that ablation of all CCAP-neurons (using *CCAP-GAL4* to express the pro-apoptotic genes *hid* and *reaper*) resulted in a failure of head eversion and leg extension at pupal ecdysis (Park et al., 2003). Here, we used a similar cell-killing approach to kill only early CCAP-neurons and test the function of late CCAP-neurons. To do this, we used the TARGET system that takes advantage of temperature-sensitive GAL80 ($GAL80^{TS}$), a yeast GAL4-repressor, to regulate GAL4/UAS activity (McGuire et al., 2004). In larvae, *CCAP-GAL4* is only expressed in early CCAP-neurons. Therefore, by raising larvae at 29°C to inactivate $GAL80^{TS}$, we could drive *UAS-hid*/*reaper* expression in early CCAP-neurons and kill them. To then prevent *UAS-hid*/*reaper* expression and cell death of late CCAP-neurons, we shifted the mid L3 larvae to 18°C so that fully functional $GAL80^{TS}$ blocked further GAL4/UAS activity. This protocol killed all early CCAP-neurons, but allowed all late CCAP-neurons to survive (n=24 animal) (**Fig. 3.2 *F***).

Remarkably, in the absence of early CCAP-neurons, the 12 late CCAP-neurons were sufficient to support normal pupal ecdysis. Head eversion and leg extension were 96% wildtype (22/23 animals) and 100% of animals eclosed (n=22) (**Fig. 3.2 *D,E***). The only observable phenotype was a 100% failure of wing inflation (n=22 animals) (**Fig. 3.2 *E***), which is known to require a subset of (ablated) early suboesophageal CCAP-INs (Davis et al., 2007; Peabody et al., 2008). As a control, we shifted animals from 29°C to 18°C at 16hr after puparium formation (APF), to repress GAL4 after the completion of pupal ecdysis. This resulted in a 100% failure of head eversion and leg extension (n=15) (**Fig 3S2 *B***). In a reciprocal test, we shifted animals from 18°C to 29°C to activate GAL4 at 15-16hr APF, so that CCAP-neurons were only killed after pupal ecdysis. We found that pupal ecdysis and eclosion were wildtype but wing inflation failed in 100% of animals (n=12) (**Fig 3S2 C, *C'***).

We then started to terminate Hid/Reaper expression (a 29°C to 18°C shift) in early pupae before pupal ecdysis. Intriguingly, we observed four primary phenotypic categories 1) A lethal failure of pupal ecdysis (n=36) (**Fig 3S2 *B***). 2) Wildtype pupal ecdysis and eclosion (n=27) (**Fig 3S2 *A***). 3) Failure of head eversion, but normal leg extension (n=9) (**Fig 3S2 *E***). 4) Failure of leg extension, but wildtype head eversion (n=20) (**Fig. 3S2 *D***). The unexpected finding that head eversion and leg extension could be uncoupled suggested that these processes are regulated by distinct CCAP-neuronal subsets. We examined the distribution of surviving CCAP-neurons in each category. Early CCAP-neurons were fully ablated in all categories. However, in category

(1), all late CCAP-neurons were also ablated, but in category (2) all late CCAP-neurons survived. Interestingly, in (3; failed leg extension, wildtype head eversion) most late A5-A7 CCAP-ENs were ablated but CCAP-PLs survived (**Fig. 3S2 *E'***). Conversely, in (4; failed head eversion, wildtype leg extension), all late A5-A7 CCAP-ENs survived but CCAP-PLs were mostly ablated (**Fig. 3S2 *D'***). Thus, late CCAP-ENs are required for leg extension whereas late CCAP-PLs are required for head eversion.

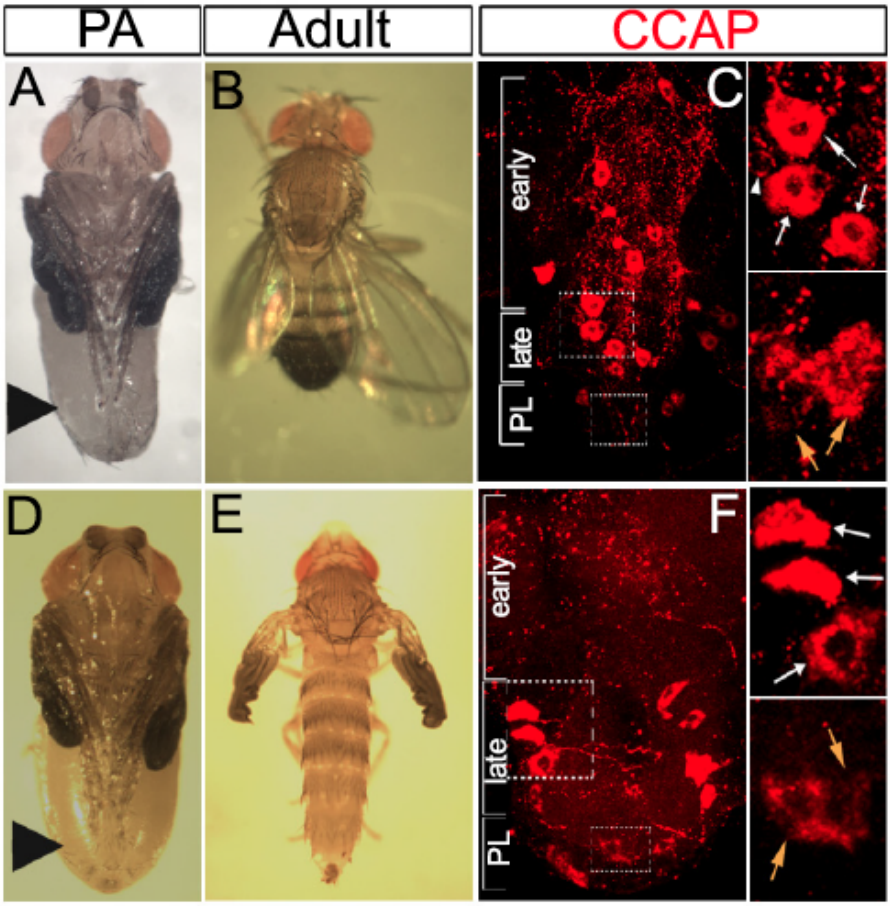

Figure 3.2. Late CCAP neurons are sufficient for pupal ecdysis

After selective early CCAP-neuron ablation, late CCAP-neurons were sufficient for pupal ecdysis. (**A,B**) Control animals exhibit wildtype leg extension at PA (arrowhead) (A), and adults have wildtype wing inflation (B). (**D,E**) Selective ablation of early CCAP-neurons results in wildtype leg extension at PA (arrowhead) (D), but adults have failed wing inflation (E). (**C,F**) CCAP expression (red) in abdominal VNC as well as close-ups of boxed regions. (**C**) In controls, the full complement of CCAP-neurons was seen; early CCAP-ENs (Long arrows), CCAP-INs (arrowhead), late CCAP-ENs (short arrows) and CCAP-PLs (orange arrows) were all observed. (**F**) All early CCAP-ENs and CCAP-INs were ablated. Only late CCAP-EN (short arrows) and CCAP-PLs (orange arrows) remained. **Genotypes.** (**A-C**) *CCAP-GAL4/+; tubP-GAL80^{TS} ,UAS-nEGFP/+* (**D-F**) *UAS-hid,UAS-reaper/+* or Y; *CCAP-GAL4; tubP-GAL80^{TS} ,UAS-nEGFP/+* .

3.4.2 Addition of late CCAP-neurons

How do late CCAP-ENs arise? Post-embryonic neuroblast lineages generate newborn neurons after mid L3 that are recruited into existing or adult-specific circuits (Lee et al., 1999). Alternatively, a differentiation switch in pre-existing neurons could occur, as for photoreceptors in Bolwig's organ that change their rhodopsin gene expression (hence light sensitivity) from a larval to an adult form (Sprecher and Desplan, 2008). To start testing these models, we examined the fate of 'early' CCAP-neurons by permanently marking them in larvae and tracking them in pharates. We raised animals of genotype *Act-FRT>STOP>FRT-nLacZ, UAS-Flp; CCAP-GAL4,UASnEGFP; tubP-GAL80^{TS}*. Here, Flp-mediated *cis-FRT* recombination brings lacZ under the actin promoter in *CCAP-GAL4*-expressing neurons, but only at 29^{o}C when GAL80^{TS} is inactive. We raised animals at 29^{o}C to mid L3 and then switched to 18^{o}C, and in pharates we found that β-Gal was expressed in all CCAP-INs and in only the anterior-most CCAP-ENs, corresponding to segments T3-A4. Notably, neither the posterior-most CCAP-ENs (the late subset corresponding to A5-A7) (**Fig. 3.3** ***A,B***) nor the CCAP-PLs expressed β-Gal (**Fig. 3.3** ***C,D***). In control experiments, we found that all early CCAP-neurons had β-Gal expression by mid L3 (**Fig. 3S3** ***A,B***), and when animals were raised at 29^{o}C throughout all development, we found that all early and late CCAP-neurons had β-Gal expression (**Fig. 3S3** ***C,D***). Thus, late CCAP-neurons did not derive from neurons that express *CCAP-GAL4* prior to late L3.

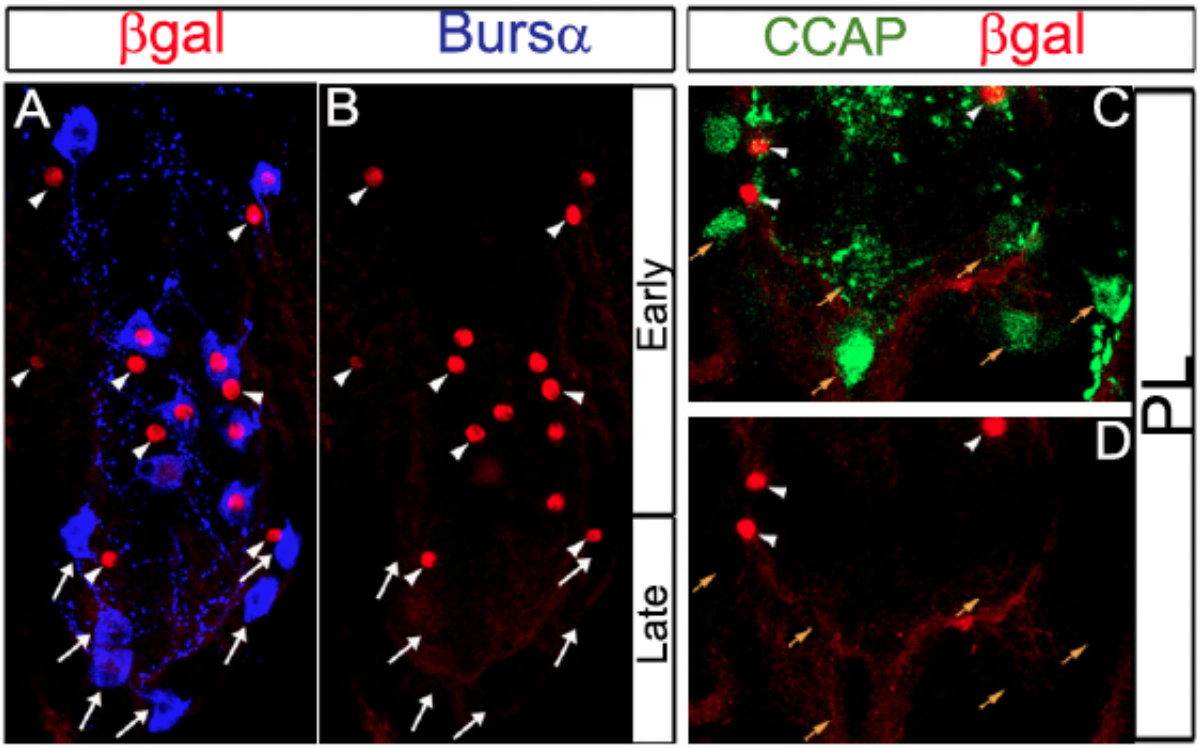

Figure 3.3. Late subsets of CCAP-neurons emerge at late L3

Combining the TARGET and Flp/FRT systems, we permanently β-Gal-marked neurons that expressed *CCAP-GAL4* in larvae. (**A-D**) Animals were raised at 29°C until mid L3 and incubated at 18°C thereafter. Anti-β-Gal (red) was not observed in either late CCAP-ENs (arrows) or CCAP-PLs (orange arrows) but was seen in CCAP-INs (arrowhead) and early CCAP-ENs (Bursα and ß-Gal co-labeled). (**A,B**) Abdominal VNC at PA. Embryonic A1-A4 CCAP-ENs (Bursα^{+ve} in blue) all co-expressed ß-Gal and Bursα, but late A5-A7 CCAP-ENs did not have ß-Gal. (**C,D**) Posterior abdominal VNC showing that CCAP-PLs (orange arrows) expressed anti-CCAP (green) but did not express ß-Gal (red). CCAP-INs (arrowheads) expressed anti-CCAP and ß-Gal. **Genotypes.** *CCAP-GAL4 /Act-FRT>STOP>FRT-nlsLacZ; tubP-GAL80^{TS}, UAS-nEGFP /UAS-Flp*

3.4.3 Late CCAP-ENs emerge by late L3

We next tested whether late CCAP-neurons derive from post-embryonic lineages. We examined BrdU incorporation into late CCAP-neurons after BrdU exposure between early L1 to pupariation. However, despite robust BrdU incorporation into many neurons, there was no incorporation into late CCAP-neurons (**Fig.S3.4 *A***). Thus, we turned to an alternate hypothesis; late CCAP-neurons derive from embryonic neuroblast lineages but undergo delayed differentiation immediately prior to pupal ecdysis.

We examined the precise timing of peptide hormone induction in late CCAP-neurons (**Fig 3. 4; Fig 3S5**). Between late L3 and pharates, Bursα and CCAP were observed in late CCAP-ENs in segments A5-A7, identified by coexpression of *OK6-GAL4*, Dac and pMad (shown for 10hr APF in **Fig 3S5 *Aii;* Fig 3.4 *Aii, Bii***). By 11hr APF at the onset of pupal ecdysis (12hr APF), these neurons also exhibited Bursβ immunoreactivity (shown for 24hr APF; **Fig 3S5 *Biii,* Fig 3.4 *Biii***). All 16 CCAP-ENs subsequently retained CCAP, Bursα and Bursβ expression (**Fig 3S5 *Aiv, Biv;* Fig 3.4 *Aiv, Biv***). The other late subset, the CCAP-PLs, had CCAP expression from late L3 pharate adults (shown for 10hr APF in **Fig 3.4 *Aii,Bii***), but did not express Bursα or Bursβ (**Fig. 3.4 *Aiv, Biv,* 1 *C* , Fig 3S1 *C,D***). CCAP-INs expressed low level CCAP up to pharate adult (**Fig 3.4 *Aiv, Biv***), but lost Bursα and Bursβ expression by 24hr PPF (**Fig 3S5 *Aiii, Biii;* Fig 3.4 *Biii***). Thus, late CCAP-neurons terminally differentiate between late L3 and pupal ecdysis to express peptide hormones that are required for pupal ecdysis at 12hr APF.

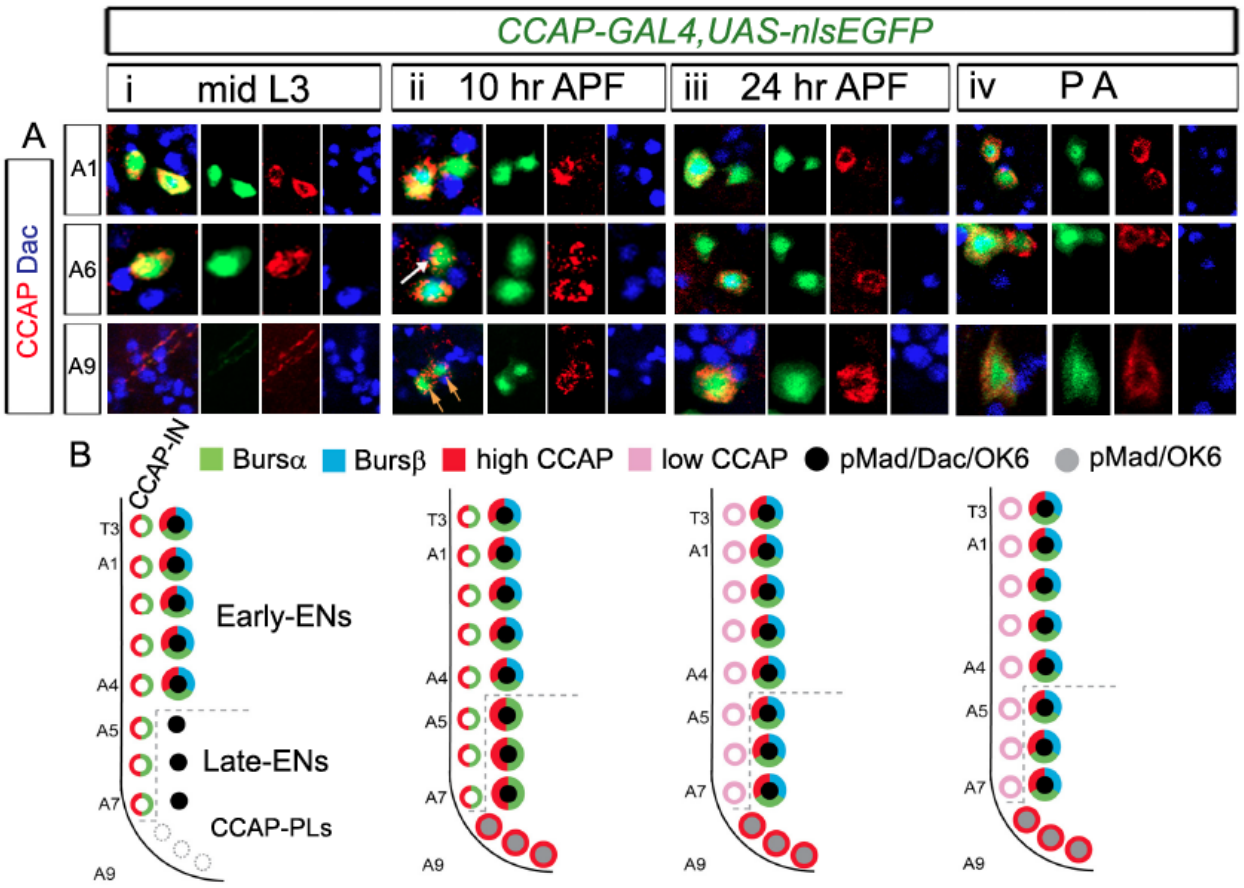

Figure 3.4. Late CCAP-neurons start to differentiate by late L3

Late CCAP-neurons started to differentiate at late L3, prior to pupal ecdysis (which occurs at 12hr after puparium formation; APF). (**A**) *CCAP-GAL4* (green), anti-CCAP (red) and Dac (blue) expression in CCAP-neurons at mid L3 before late-CCAP neuron differentiation **(i)**, at 10hr APF immediately prior to pupal ecdysis **(ii)**, at 24hr APF **(iii)**, and at pharate adult (PA) **(iv).** At each age, we show representative images of CCAP expression in hemisegments T3-A4 (A1), A5-A7 (A6), and A8-A9 (A9). (**Ai-iv;A1**) CCAP was expressed in all CCAP-INs and early CCAP-ENs from mid L3 to PA. (**Ai-iv;A6**) CCAP expression in late CCAP-ENs started at late L3 (arrow in Aii) and continued to PA. (**Ai-iv;A9**) CCAP expression in late CCAP-PLs started at late L3 (orange arrows in Aii) and continued to PA. CCAP-PL did not express Dac, Bursα or Bursβ **(B)** Cartoon summaries of gene expression in VNC CCAP-neurons from mid L3 to PA. **Genotypes:** *CCAP-GAL4,UAS-nlsEGFP/+*.

3.4.4 Late CCAP-neurons are embryonic but terminally differentiate at late L3

We previously reported that early CCAP-ENs in T3-A4 could be identified by *OK6*-GAL4, Dac and pMad co-expression (Veverytsa and Allan, 2011). Although a small number of neurons co-express these markers, CCAP-ENs are the only neurons to do so in the dorsal ventral nerve cord. Moreover, by late L3, late CCAP-ENs in A5-A7 also co-expressed the same markers. Using this discriminatory approach to examine the St17 to midL3 VNC, we were able to confirm the presence of a single dorsal neuron per hemisegment in A5-A7 that co-expressed all three markers (shown for late St17; **Fig 3.5 *A***). Intriguingly, in St15-eSt17 embryos, we observed transient, precocious Bursα expression in those A5-A7 neurons, strongly suggestive of a CCAP-EN identity (**Fig 3S4 *B,C***). To further verify that late CCAP-ENs were born in the embryo, we took advantage of their expression of Dac to Flp-in a lacZ reporter under the conditional activation of the Dac^{GAL4} reporter. We raised *Act-FRT>STOP>FRT-nLacZ,UAS-Flp; dac-GAL4,UASnEGFP; tubP-*$GAL80^{TS}$ animals at 29°C from late St17 to early L2 and then switched to 18°C thereafter. In pharates, we observed β-Gal immunoreactivity in all large CCAP-ENs, showing that the six late CCAP-ENs in A5-A7 were born and express Dac in the embryo (**Fig 3.5 *B***). These data further suggest that CCAP-ENs are generated in all segments T3-A7, but that in segments A5-A7, a mechanism exists that delays CCAP-EN terminal differentiation. Lack of BrdU incorporation into CCAP-PLs led us to postulate that CCAP-PLs are also born in the embryo (**Fig 3S4 *A***). A lack of discriminatory markers for CCAP-PLs in the embryo precluded their direct identification. However, we took advantage of their expression of *OK6-GAL4* (a postmitotic marker) to cell-selectively Flp-in a lacZ. We raised *Act-FRT>STOP>FRT-nLacZ,UAS-Flp; OK6-GAL4,UASnEGFP; tubP-*$GAL80^{TS}$ animals at 29°C from late St17 to late L1 and then switched to 18°C thereafter. In pharate adults, we observed β-Gal immunoreactivity in most CCAP-PLs (**Fig 3S1 *D***), showing that CCAP-PLs were born and express *OK6-GAL4* in the embryo.

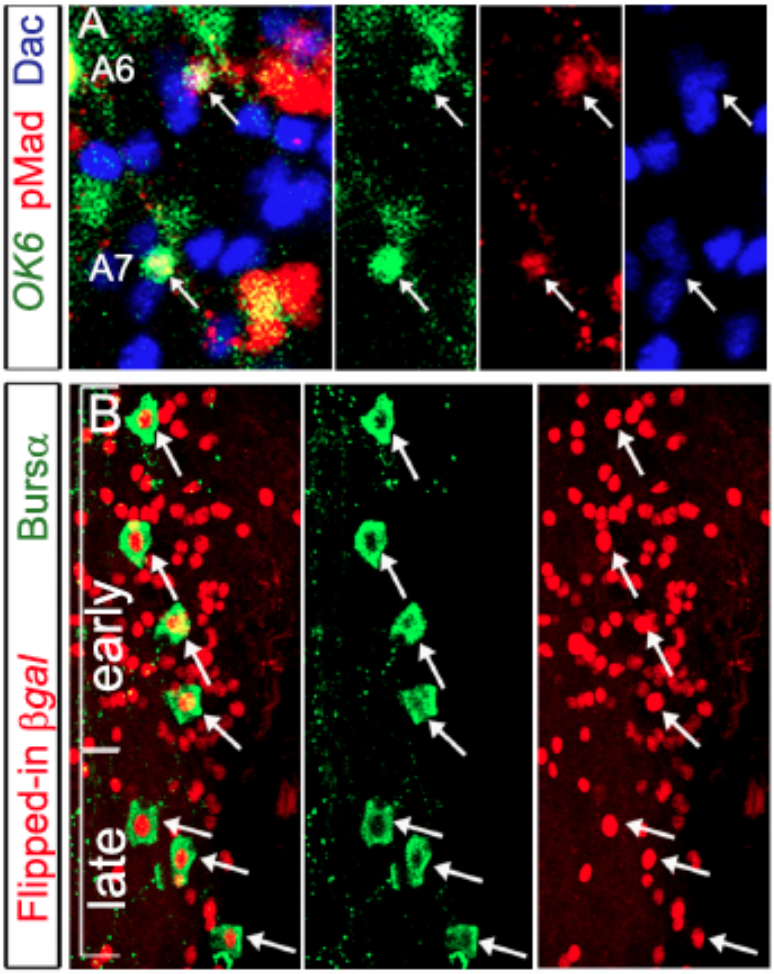

Figure 3.5. Late CCAP-ENs derive from an embryonic lineage

Postmitotic late CCAP-ENs can be observed at late embryonic stages. **(A)** Triple label and fluorophore splits shown for hemisegments A6 and A7. Embryonic stage 17, dorsal-half VNC showed that a single dorsal neuron (arrow) in each hemisegment triple-labeled for pMad (red), *OK6-GAL4* (green) and Dac (blue). This overlap is unique to CCAP-EN in the dorsal half VNC in hemisegments T3-A4 (Veverytsa and Allan, 2011). (**B**) Pharate adult abdominal region of VNC (with fluorophore splits) labeled for β-Gal (red) and Bursα (green). Early and late CCAP-neuron subsets demarcated. Using Dac^{GAL4} with TARGET and Flp/FRT, we raised animals from late St17 to early L2 (prior to post-embryonic neurogenesis) and switched to 18°C thereafter. In pharate adults, anti-β-Gal was observed in all CCAP-ENs (arrows) in hemisegments A1-A7. Thus, all six late CCAP-ENs in A5-A7 were born and expressed Dac between late St17 and early L2. **Genotypes:** (**A**) *OK6-GAL4, UAS-nlsEGFP/+*; (**B**) dac^{GAL4}*/Act-FRT>STOP>FRT-nlsLacZ; tubP-GAL80*^{TS}*, UASnEGFP /UAS-Flp*

3.4.5 The ecdysone cascade drives late CCAP-neuron differentiation

The ecdysone-induced nuclear hormone receptor cascade plays a critical role in the metamorphic changes that take place during pupariation. This cascade promotes post-embryonic neurogenesis, remodeling of neuronal morphology and in certain cases, changes in neuronal gene expression (Schubiger et al., 1998; Winbush and Weeks, 2011). We tested the cell-autonomous role of this cascade in temporally inducing late CCAP-neuron differentiation.

We used *CCAP-GAL4* to cell-specifically drive isoform-specific blockers of the ecdysone receptor and found that dominant negative transgenes to EcR-A (EcR-A^{DN}) (**Fig 3.6 *B'***) or EcR-B1 (EcR-$B1^{DN}$) (**Fig 3.6 *C'***) blocked late CCAP-ENs differentiation. In controls, CCAP immunoreactivity was observed in 14.7±1.7 CCAP-ENs per VNC (n=10 VNCs). EcR-A^{DN} or EcR-$B1^{DN}$ expression reduced this to 6.4±1.1 CCAP-ENs (n=9, P<0.0001) and 5.8±2.4 CCAP-ENs (n=8, P<0.0001) respectively. Importantly, expression was only retained in the anterior-most CCAP-ENs of T3-A4. In controls, Bursα expression was observed in 14.7±1.7 CCAP-ENs per VNC (n=10 VNCs). EcR-A^{DN} or EcR-$B1^{DN}$ reduced this to 7.3±1.2 CCAP-ENs (n=6, P<0.0001) and 6.7±1.2 CCAP-ENs (n=6, P<0.0001), respectively. Finally, in controls, Bursβ expression was observed in 14.1±1.5 CCAP-ENs per VNC (n=10 VNCs). EcR-A^{DN} or EcR-$B1^{DN}$ reduced this to 7.7±3.0 CCAP-ENs (n=10, P<0.0001) and 6.7±1.8 CCAP-ENs (n=9, P<0.0001), respectively. In all cases, peptide hormone expression was lost exclusively in the posterior-most, or late, subset of CCAP-ENs. These data correspond well with pupal ecdysis phenotypes we observe in these animals; expression of either *UAS-EcR-A^{DN}* (**Fig 3.6 *B***) or *UAS-EcR-$B1^{DN}$* (**Fig 3.6 *C***) resulted in a failure of leg extension whereas *UAS-EcR-$B2^{DN}$* had no effect (**Fig 3.6 *D***). Intriguingly, ecdysone receptor manipulation had no apparent effect on the expression of CCAP in late CCAP-PLs in pupae. This lack of effect may reflect the redundancy for EcR-B1/A function in CCAP-PLs or may reflect the significantly weaker expression of *CCAP-GAL4* (and thus lowered expression of the EcR-DN constructs) that we have found in those neurons compared to CCAP-ENs. However, these data may explain the weak head eversion phenotype upon EcR-$B1^{DN}$/A^{DN} expression that we observe, given our results that demonstrate the specific role of CCAP-PLs in directing head eversion (above).

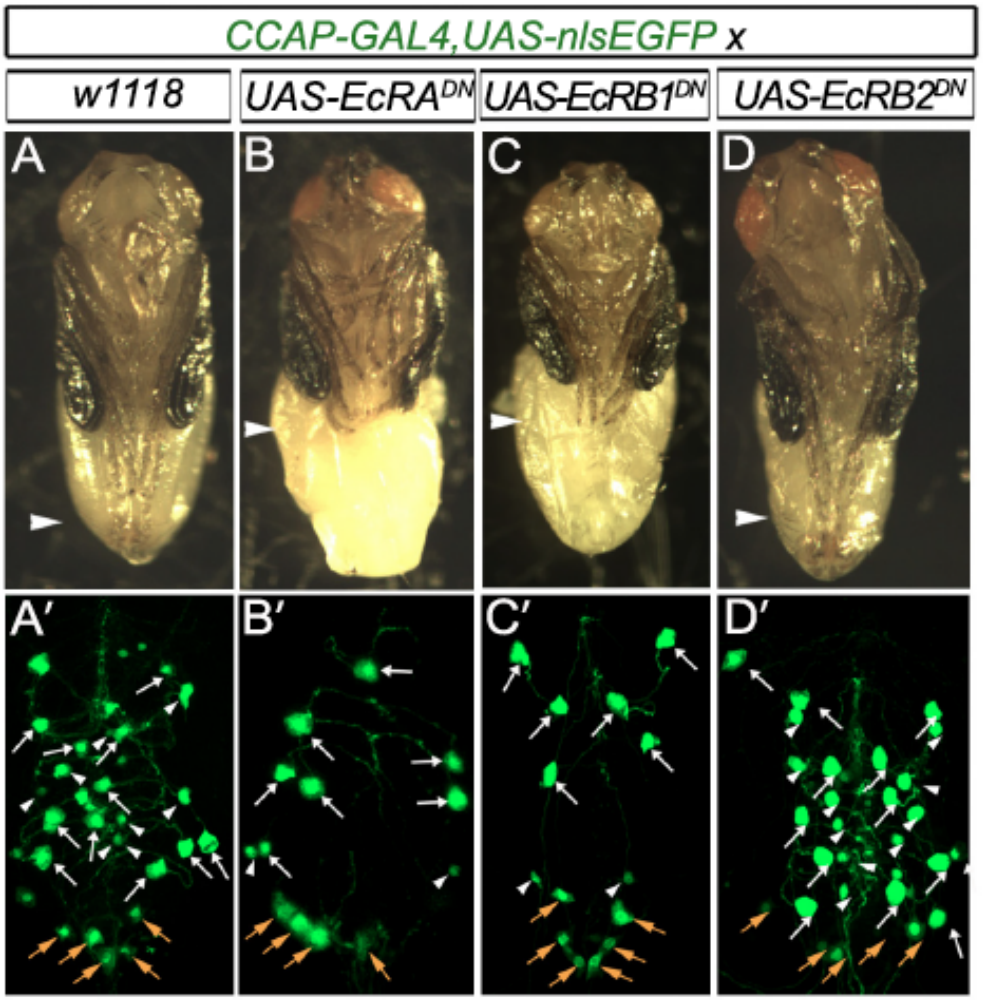

Figure 3.6. Ecdysone receptors A and B1 are required for late CCAP-EN differentiation

CCAP-GAL4-mediated overexpression of dominant-negative EcR-B1/A in late differentiating CCAP-ENs shuts down further differentiation. **(A-D)** The extent of leg extension in pharate adults (PA) is shown by arrowhead for each genotype. Control (A) and *EcR-B2*^{DN} flies (D) had wildtype leg extension, but overexpression of dominant-negative *EcR-A*^{DN} (**B**) or *EcR–B1*^{DN} (**C**) resulted in failed leg extension. **(A'-D')** Pharate adult abdominal VNC showing distribution of all *CCAP-GAL4,UAS-nEGFP*-labeled neurons (green). In controls (**A'**) and *EcR-B2*^{DN} (**D'**), all CCAP-ENs (arrows), CCAP-INs (arrowheads) and CCAP-PLs (orange arrows) expressed *CCAP-GAL4*. In *EcR-A*^{DN} (**B'**) or *EcR–B1*^{DN} (**C'**) flies, late CCAP-ENs lose *CCAP-GAL4* expression. Early CCAP-ENs (arrows) and CCAP-PLs (orange arrows) were unaffected. **Genotypes:** (**A,A'**) *CCAP-GAL4, UAS-nEGFP/+*; (**B,B'**) *CCAP-GAL4, UAS-nEGFP/UAS-EcR-A*^{DN} ; (**C,C'**) *CCAP-GAL4, UAS-nEGFP/UAS-EcR-B1*^{DN}; (**D,D'**) *CCAP-GAL4, UAS-nEGFP/UAS-EcR-B2*^{DN} .

We examined the role of *ftz-f1* due to its central role in the ecdysone-induced nuclear hormone receptor cascade. Due to the embryonic lethality of *ftz-f1* null mutants, we attempted to examine hypomorphic *ftz-f1* mutants for late CCAP-neuron differentiation. However, as we found that 89% of mutants died prior to pupal ecdysis, we turned to gain of function studies. Remarkably, a 1hr induction of *ftz-f1* (using *ftz-f1* expressed from a heat shock promoter induced at 37^{o}C) in late L1 larvae resulted in precocious differentiation of late CCAP-ENs and CCAP-PLs within four hours. We observed precocious Bursα immunoreactivity in late CCAP-ENs in 54% of animals (n=13 animals), Bursβ in 40% of animals (n=20) and CCAP in 38% of animals (n=24) (shown for Bursα **Fig 3.7 *A,B***). Precocious CCAP expression was also observed in CCAP-PLs and, appropriately for CCAP-PLs, neither Bursα nor Bursβ. Thus, precocious induction of *ftz-f1* induced activation of appropriate subtype-specific sets of peptide hormones in late CCAP-neurons.

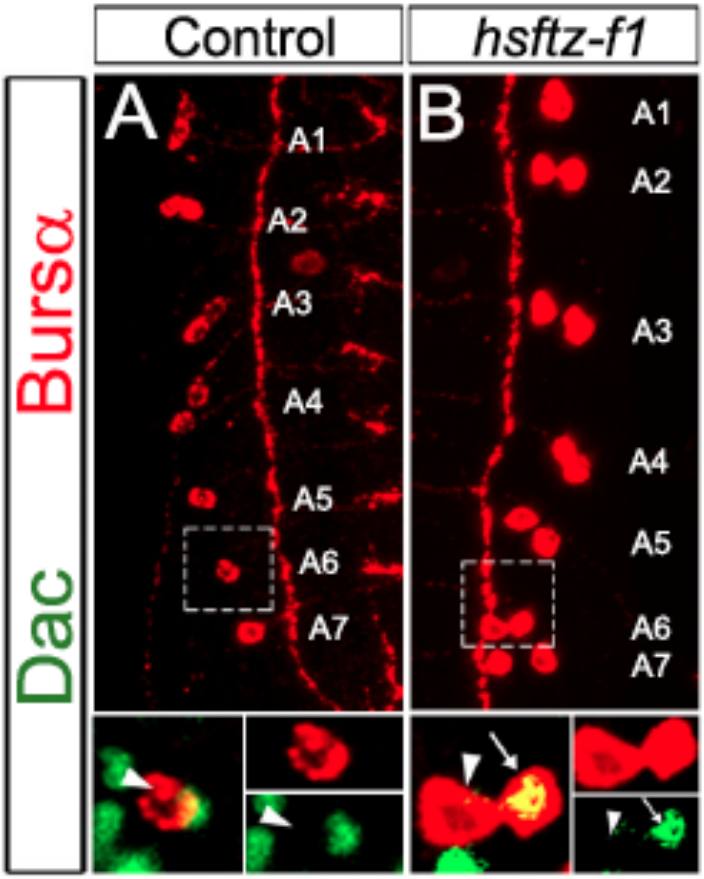

Figure 3.7. Overexpression of *ftz-f1* precociously differentiates late CCAP-ENs

(**A**) Control early L2 dorsal-half VNC double-labeled (with fluorophore splits of boxed area) for Bursα (red) and Dac (green), in T3-A7 hemisegments. CCAP-neuron doublets expressing Bursα were only observed in hemisegments T3-A4. Boxed region shows that in A5-A7 only Dac^{-ve} CCAP-INs had differentiated to express Bursα (arrowhead). (**B**) *hs-ftz-f1* animals were heat shocked at late L1. CCAP-neuron doublets expressing Bursα were observed in each hemisegment T3-A7. Boxed region shows closeup of A6 hemisegment. The additional CCAP-neuron (arrow) expressed Dac, indicative of precocious late CCAP-EN differentiation. Similar results were obtained for precocious activation of CCAP and Bursβ in CCAP-ENs, and precocious CCAP expression in CCAP-PLs (see text for details). **Genotypes**: (A) *CCAP-GAL4,UAS-nlsEGFP/+*; (B) *hsbftz-f1/ hsbftz-f1*.

3.5 DISCUSSION

We show that the CCAP-neuron network recruits two additional neuronal subsets immediately prior to pupal ecdysis, which are sufficient for pupal ecdysis. We further show that late CCAP-ENs specifically direct appendage extension while late CCAP-PLs direct head eversion. Intriguingly, unlike other well-established models in which post-embryonic neurogenesis adds newborn neurons to existing larval networks at metamorphosis, we find that temporally-tuned terminal differentiation provides the mechanism of functional neuronal recruitment to an existing network, and show that this can support a change in network behavioral output. The temporally-tuned differentiation of peptide hormone gene expression in late CCAP-neurons is of critical relevance, as these peptide hormones are the primary output of CCAP-neurons that coordinate the behavioral sequence of ecdysis (Ewer, 2005; Kim et al., 2006a; Veverytsa and Allan, 2011). Together, these data highlight an unanticipated complexity in the functional heterogeneity of the CCAP-neuronal network (Luan et al., 2006a) and outline the regulatory mechanisms and functional relevance of temporally-tuned terminal differentiation in remodeling a functioning neuronal network.

3.5.1 Temporally-tuned differentiation of postmitotic neurons

A neuron is typically not considered to be terminally differentiated until it expresses the terminal differentiation genes required for its function. However, neuronal differentiation is not a singular event, but often a protracted process. For example, axon pathfinding genes are typically induced upon axon outgrowth but many genes critical to function such as neuropeptides, ion channels and those implicated in network connectivity can be induced later in development (Allan et al., 2003; Martin-Caraballo and Dryer, 2002; Vrieseling and Arber, 2006). Most embryonic *Drosophila* neurons fully differentiate by larval stages (Tissot and Stocker, 2000), but examples of delayed differentiation have emerged. Most motoneurons are born in the embryo and function in the larva, to then be remodeled during metamorphosis and re-used to innervate adult muscles (Tissot and Stocker, 2000). However, the mesothoracic motoneuron, MN5, projects short immature processes during embryogenesis but then developmentally arrests until metamorphosis, whereupon it elaborates mature arbors and innervates its muscle target (Consoulas et al., 2002). The functional relevance of this delayed-onset morphological

differentiation is evident, but the underlying gene regulatory mechanisms are unknown. Also, Tv2/3 neurons are born as part of the Tv cluster in thoracic segments T1-T3 during embryogenesis, but unlike the Tv1 and Tv4 neurons that differentiate into Nplp1 and FMRFa-expressing neurons in the embryo (Baumgardt et al., 2007; Benveniste et al., 1998), Tv2/3 neurons do not differentiate into FMRFa-expressing neurons until metamorphosis (Schneider et al., 1993a; Schneider et al., 1993b). Work in *Manduca sexta* has reported an increase in the number of CCAP-immunoreactive neurons in abdominal segments A2-A7 by day 1 of the fifth Instar, prior to pupal ecdysis. Backfill experiments suggested that these neurons were present in the 3rd and 4th instars, but did not differentiate until the 5th Instar (Dai et al., 2008; Davis et al., 1993; Loi et al., 2001). However, it was unknown whether these 'late'-appearing CCAP-neurons were of embryonic or post-embryonic origin, nor if they had a functional role in pupal ecdysis. Work presented here addresses these questions in *Drosophila*.

3.5.2 Late CCAP-ENs are poised for ecdysone-dependent neuronal differentiation

The role of ecdysone signaling in the metamorphic changes of the insect nervous system is well established, such as in neuronal programmed cell death, post-embryonic neurogenesis and morphological remodeling (Robinow et al., 1993; Schubiger et al., 1998; Weeks and Levine, 1990). Ftz-f1 is an important downstream regulator of the ecdysone nuclear hormone cascade (King-Jones and Thummel, 2005), but less well studied for its role in neurons. Previous studies demonstrated that *ftz-f1* dictates the temporal specificity of ecdysone signaling by integrating messages between numerous nuclear receptors, E75, DHR3, DHR4 and potentially also E78 and DHR39 (Broadus et al., 1999; Woodard et al., 1994). In spite of its long-known expression in the nervous system, the only reported role for *ftz-f1* in nervous system metamorphosis, to our knowledge, is in remodeling mushroom body neuron morphology (Boulanger et al.; Ohno and Petkovich, 1993; Ruaud et al., 2010; Yamada et al., 2000). Here, we uncover an important role for *ftz-f1* in triggering timed neuronal differentiation. It will be illuminating in the future to determine whether EcR and Ftz-f1 directly or indirectly regulate peptide hormone expression in late CCAP-neurons.

Currently, it is unclear why late CCAP-neurons would undergo timed differentiation. We previously found that CCAP-ENs are important to both larval and pupal ecdysis (Veverytsa and Allan, 2011). However, larval and pupal ecdyses are behaviorally distinct, and here we find that late CCAP-ENs are undifferentiated at larval ecdysis but entirely sufficient amongst CCAP-

neurons for pupal ecdysis. We postulate that the emergence of a late subset of CCAP-ENs immediately prior to pupal ecdysis offers an elegant means to meet the changing functional role of the CCAP-neuronal network from larval to pupal ecdysis. We propose three mechanisms that may explain why the late CCAP-ENs are recruited for pupal ecdysis but not larval ecdysis. 1) Late CCAP-ENS may have distinct central connectivity or receptor expression that may mediate a difference in the sequence or activation of CCAP-neuron activity that is specific to pupal ecdysis. These differences may also make early differentiation of late CCAP-ENs detrimental to larval ecdysis. 2) Early CCAP-ENs on their own may not secrete sufficient peptide hormone levels into the haemolymph for robust pupal ecdysis and late CCAP-ENs may be required to boost levels of secreted peptide hormones required for pupal ecdysis, but not larval ecdysis. 3) The temporally-tuned differentiation mechanism may have evolved simply to conserve the demands of secreting high levels of circulating peptide hormones until the timepoint at which it is absolutely required.

A delay in the differentiation of embryonic neurons may offer a simpler mechanism to generate late CCAP-neurons than by post-embryonic neurogenesis. The process of neuronal specification and differentiation is a highly orchestrated process through which complex cascades of spatially and temporally patterned transcription factors direct the emergence of specific neuronal subsets. Moreover, the *Drosophila* nervous system is segmented, with abdominal segments A1-A7 giving rise to essentially the same neuroblasts and mostly the same neurons in each segment. Thus, generating a single CCAP-EN in each segment from the same neuroblast lineage would be an economical way to produce late CCAP-ENs with much the same ultimate gene expression profile as early CCAP-ENs. But, clearly, additional mechanisms for segment-specific diversity must exist to distinguish early from late postmitotic CCAP-ENs. Recent work has described such mechanisms. The best described of these is the programmed cell death of postmitotic neurons in specific segments (Miguel-Aliaga and Thor, 2004; Suska et al., 2011). These studies indicate that segment-specific differences in Hox gene expression within specific postmitotic neuronal subsets determine their survival versus apoptosis. Here, we have uncovered that segment-specific differences in the timing of neuronal differentiation provides a novel mechanism for neuronal subset diversity. It will be intriguing in the future to investigate the potential role of differential Hox gene expression in early versus delayed differentiation of the CCAP-neuron population.

4. CONCLUSION

4.1 OVERVIEW OF RESEARCH UNDERTAKEN

This thesis aimed to relate the differentiation of terminal gene expression in neurons to the functional output of a neuronal network. Specifically, we attempted to identify gene regulatory mechanisms within the CCAP-neuronal network that had an impact on the function of CCAP-neurons in regulating ecdysis. Focusing on the CCAP-neuron network had a number of advantages: 1) CCAP-neurons co-express four identified peptide hormone TDGs that are effectors of CCAP-neuron function. 2) We can manipulate gene function in this neuronal population *in vivo*. 3) Disruption of normal development of this neuronal population produces an easily assayed behavioral phenotype.

With these combined advantages, the strengths and advances of the work presented in this thesis have been: 1) To demonstrate how neuronal differentiation, and ultimately the function of the neuronal network, can be controlled by factors extrinsic to the neurons themselves (BMP and ecdysone signaling). 2) To demonstrate that temporally-tuned differentiation of specific neuronal subsets is utilized within a functioning network as a developmental mechanism to support changes in behavioral output.

Overall, in addition to these advances in the field of neuronal differentiation, this work provides a foundation and a model for future efforts to dissect the gene regulatory mechanisms that act within neuronal networks to ensure appropriate network function. In this conclusion, I will tie together the findings of the two manuscripts that form the body of this thesis, as well as discuss the overall significance of the work and directions that could be taken in the immediate future to build upon this work.

4.2 INTEGRATING THE FINDINGS OF THIS THESIS WORK, AND ITS SIGNIFICANCE TO THE FIELD

4.2.1 Advancing our understanding of CCAP-neuron network function

The function and gene expression of CCAP-neurons has been studied in *Drosophila* and *Manduca sexta* for decades (Davis et al., 1993; Honegger et al., 2008; Kim et al., 2006a; Kim et

al., 2006b; Park et al., 2003). The CCAP-neuron network has been well-studied for three primary reasons (discussed in details in Section 1.7 of the Introduction) ; i) the agricultural potential in pest control of studying CCAP-neurons due to their function in insect ecdysis and wing inflation, ii) the clear phenotypic consequences of disrupting these neurons have made the CCAP-neurons a screenable tool for basic neurological and biological principles (Bohm et al., 2010; Peabody et al., 2009; Zhao et al., 2008), and iii) their participation in a complex neuropeptide and peptide hormone cascade. Several key findings have emerged from previous work relevant to this thesis. Focusing on *Drosophila*, these are: 1) Ewer and colleagues found that ablation of all CCAP-neurons slowed the progress of larval ecdysis and entirely blocked pupal ecdysis (Park et al., 2003). 2) The Hewes lab showed that overexpressing dominant negative form of ecdysone receptor B1 results in defects in pupal ecdysis (Zhao et al., 2008). 3) Ben White and colleagues had found that CCAP-neurons are subdivided into distinct functional subsets. By genetic dissection of the network, only the subesophageal CCAP-interneurons (-INs) and the CCAP-efferent neurons (-ENs) were found to be required for wing inflation (Luan et al., 2006a). 4) Adams and colleagues showed that specific subsets of CCAP-neurons are activated by ETH at the time of pupal ecdysis (Kim et al., 2006b). The work in this thesis has build upon this foundation to answer a number of previously unaddressed questions: 1) Which subsets regulate ecdysis? This thesis has outlined the central role played by CCAP-ENs and CCAP-PLs in ecdysis, and provides the unexpected finding that all early CCAP-neurons are dispensable for pupal ecdysis, in the presence of late CCAP-neurons. 2) How is peptide hormone gene expression regulated in CCAP-neurons? This thesis has defined, in detail, the expression profile of all peptide hormones in CCAP-neurons, and has identified BMP signaling, ftz-f1 and EcR as key regulators of CCAP-neuron peptide hormone expression. These studies also demonstrated key differences in the regulation of these peptide hormones within different subsets of the CCAP-neuronal population. 3) What is the origin of the additional CCAP-ENs that had been observed in pharate adults? Our studies found that an additional set of CCAP-neurons differentiate during late larval stages and play an important role in pupal ecdysis. These then remain to the pharate stage to contribute to the CCAP-EN population at that time.

Together, the findings presented in this thesis have added to the established model of CCAP-neuron gene regulation and function to provide a better general understanding of neuronal function and remodeling with regards to network output.

4.2.2 Role of extrinsic signaling in neuronal differentiation

There has been increasing evidence that neurons are not hard-wired by their lineage but are under significant regulation from extrinsic influences. It is important to note that even though this concept is well established in vertebrate models, a long-standing assumption had been that the differentiation of invertebrate neurons, such as in *C.elegans* and *Drosophila*, were largely hard-wired depending on their lineage. One of the first studies challenging this concept was the demonstration of target-derived neuronal differentiation in *Drosophila* by the BMP pathway (Allan et al., 2003).

The work of this thesis has continued this line of investigation and uncovered how retrograde BMP signaling as well as the ecdysone cascade both integrate into CCAP-neurons to control the expression of peptide hormones, and ultimately the function of the entire network. We have shown how retrograde BMP signaling controls *Drosophila* behavior via regulation of multiple co-expressed peptide hormones within a specific neuronal subset. This is the first example to show that BMP-dependent gene expression in a neuronal subset underlies the execution of a patterned behavior. Further, we show that retrograde BMP signaling in *Drosophila* CCAP-neurons differentially regulates numerous genes in individual neurons. Together with previous work, we have now identified five peptide hormone genes that are expressed in specific subset of neurons, and regulated to different extents by the same retrograde BMP signal. This now provides the basis of future studies to understand the transcriptional mechanisms through which BMP signaling regulates neuronal subset-specific gene expression. Currently, this is not understood.

Our initial analysis of BMP signaling in CCAP-neurons provided a foundation for our efforts to dissect how the CCAP-neuronal network is remodeled during metamorphosis. This analysis proved very interesting, because we were able to demonstrate the role of temporally-tuned differentiation in network remodeling, as well as provide certain details of the underlying regulatory mechanisms. Aside from well-established models showing that post-embryonic neurogenesis and morphological restructuring remodels larval neuronal circuits to reshape functional output (Brown and Truman, 2009; Lee et al., 1999; Levine et al., 1995; Zheng et al., 2003), few other mechanisms have been identified that remodel neuronal networks during metamorphosis. First, the Desplan lab has shown a unique example of fate switching in the photoreceptors of Bolwig's organ. They found that a set of photoreceptors of the larval light detector, Bolwig's organ, switched their expression of rhodopsin genes from Rh5 to Rh6 during

metamorphosis. This changed their functional light sensitivity from blue (Rh5) in larvae to green (Rh6) in adults. (Sprecher and Desplan, 2008). Second, examples of delayed differentiation have been identified for subsets of motoneurons (MN5) and Tv neurons (Tv2/3). However, the regulatory mechanisms for delayed differentiation had not been identified. One interesting finding of our studies was the identification of the role of ftz-f1 in temporally-tuned differentiation. Previous work on ftz-f1 led to the conclusion that ftz-f1 is not involved in neuronal specification (Boulanger et al., 2011). In this thesis, our gain-of-function analysis showed that ftz-f1 acts as (part of) the temporal mechanism that mediates temporally-tuned differentiation of late CCAP-neurons. It would be interesting to revisit the other examples of delayed differentiation or the rhodopsin gene switch in Bolwig's organ to test for a putative role of ftz-f1.

4.3 LIMITATIONS AND FUTURE DIRECTIONS OF THE DISSERTATION

We have shown the first link between BMP-dependence of multiple co-expressed peptide hormones and behavior, as well as how a functioning neuronal network utilizes delayed terminal differentiation to remodel functioning neuronal networks. However, the work in this dissertation had a number of limitations.

In chapter 2, we performed cell-autonomous peptide hormone rescue in BMP mutants in all CCAP-neurons, due to a lack of CCAP-neuron subset-specific drivers. This is one of the limitations throughout this dissertation – the lack of GAL4 drivers highly specific to small subsets of CCAP-neurons. This meant that we were rescuing peptide hormone levels in all CCAP-neurons, not just CCAP-ENs, even though loss of BMP signaling only affected peptide hormone in the CCAP-EN subset of CCAP-neurons. One additional limitation, that would be an issue with any use of the GAL4/UAS system, is that we could not precisely rescue the normal amount of peptide hormone into CCAP-neurons in these experiments. This creates a problem because we were not able to rescue peptide hormones at physiologically normal levels. However, our experiments did produce a significant rescue of pupal ecdysis, in spite of probably overexpressing peptide hormones. This leaves open the possibility that BMP signaling affects other processes in CCAP-neurons that are not related to peptide hormone expression. For example, one possibility is the process of peptide hormone secretion. It is currently unknown whether retrograde BMP signaling regulates peptide hormone secretion. However, reduced neurotransmitter secretion is well characterized in BMP-mutant neuromuscular junctions (Aberle et al., 2002; Marques et al., 2002), making it possible that peptide hormone secretion is also

affected. Thus, we cannot formally state that loss of peptide hormones expression is the only way in which loss of BMP signaling disrupts CCAP-neuron function to cause defects in larval and pupal ecdysis.

In chapter 3, we identified two distinct late subsets of CCAP-neurons, the CCAP- ENs and the previously unidentified CCAP-PLs. By killing CCAP-neurons at different timepoints we were able to distinguish the functional sufficiency of these two subsets. Unfortunately, we were unable to selectively kill or electrically-inactivate either the late CCAP-ENs or CCAP-PLs, or both, while leaving the early CCAP-neurons intact. Thus, similar to the limitation encountered in chapter 2 (above), in chapter 3, subset-specific GAL4 drivers would have allowed us to test the necessity of late CCAP-ENs in pupal ecdysis and further allow us to dissect the network. Currently, we are attempting to overcome this limitation by building sets of transgenic flies that can address this issue. However, despite this limitation, we have shown that late CCAP-ENs are sufficient for pupal ecdysis and that different subsets of late CCAP-neurons have distinct functions in pupal ecdysis. These data also correspond to previous findings by the Adams group that found that different subsets of CCAP-neurons are activated during different stages of pupal ecdysis (Kim et al., 2006b).

In the ftz-f1 overexpression experiments of chapter 3, we were able to show that larval induction of ftz-f1 expresison precociously activated differentiation of late CCAP-neurons. Thus, we concluded that ftz-f1 plays an important role in temporally-tuned differentiation of late CCAP-neurons. However, verification of the necessity of ftz-f1 was limited by the lack of strong ftz-f1 mutants that survived to the late L3/white pupa stage, so that we could test late CCAP-neuron terminal differentiation. In order to overcome this, we have started to utilize publicly available UAS transgenes with double-stranded RNAi to ftz-f1 to examine the effect of ftz-f1 knockdown on gene expression / differentiation of the late subset of CCAP-neurons. Many studies have pointed to ftz-f1 being part of the ecdysone-inducible cascade. However, a recent report showed that ftz-f1 plays an important role in increasing EcR expression in mushroom body neurons (Boulanger et al., 2011). Therefore, it will be interesting to resolve the epistatic relationship between EcR and ftz-f1 in relation to late CCAP-neuron differentiation.

4.3.1 Cis-regulation of gene expression in CCAP-neurons

Terminal differentiation refers to the induction of subtype-specific TDGs that carry out the neuron's function. TDG expression is induced by transcription factors acting at specific *cis*-

regulatory motifs (CRMs) in the *cis*-regulatory regions of each gene, as well as target-derived signals that presumably act via inducible transcriptional mechanisms at the same *cis*-regulatory regions (Allan et al., 2003; Ben-Tabou de-Leon and Davidson, 2007; Philippakis et al., 2006; Wenick and Hobert, 2004). Although there has been tremendous progress in understanding how transcription factors act individually and combinatorially at CRMs, we are still far from understanding the complexities of inducing the many TDGs that are required for neuronal terminal differentiation. Also, how intrinsic and extrinsic factors combinatorially regulate TDG expression in neurons is currently unknown.

One future goal, using CCAP-neurons as a model, would be to identify the *cis*-regulatory sequences through which transcription factors determine the spatial and temporal regulation of gene expression in CCAP-neurons. In work not discussed in this thesis, we have identified short *cis*-regulatory regions for CCAP, Bursα and Bursß genes (each ~100bp) that generate the appropriate *in vivo* spatial and temporal expression of each gene in CCAP-neurons. The identification of these *cis*-regulatory regions now form a foundation for using CCAP-neurons as a good model for investigating how *cis*- (and *trans*-) regulatory mechanisms act in single identified neurons to ensure appropriate neuronal differentiation and function. A number of questions can be addressed using the CCAP-neuron model. First, we have analyzed these *cis*-regulatory regions using a number of publicly available on-line bioinformatic approaches exploring phylogenetic conservation and candidate transcription factor binding sites. These studies identified which of these sequences were conserved through all 12 sequenced *Drosophila* species, and hence presumably likely to be important for expression, and also provided us with a number of candidate transcriptional regulators for future testing. Second, we have generated a series of transgenes in which we have mutated (by substitution) all phylogenetically conserved sequences within these *cis*-regulatory regions. These are being assessed and categorized based on: 1) Loss or significant reduction of expression in all CCAP-neurons, indicating that they are essential CRMs for expression. 2) Ectopic expression, which would be indicative of repressor sequences for preventing expression in inappropriate cells. 3) Timing defects in expression that may show precocious expression in pre-differentiated late CCAP-ENs, or a failure of induction in late CCAP-ENs. Timing defects would be informative as to the potential mechanisms of temporally-tuned differentiation, the former suggesting an active repressor mechanism in pre-differentiated late CCAP-ENs, whereas the latter suggests a late activation model.

4.3.2 Trans-regulation of CCAP-neuron

Multiple transcription factors regulate TDG expression in both *Drosophila* and vertebrates (Allan et al., 2003; Jorgensen et al., 2004). Thus, identification of transcription factors expressed in CCAP-neurons that are essential for peptide hormone expression would be important for better understanding peptide hormone regulation, spatially and temporally. We have begun a screen to identify transcription factors essential for peptide hormone expression in CCAP-neurons by obtaining available antibodies and reporters to all known *Drosophila* transcription factors that are known to have a role in neuronal specification and differentiation. Currently, we have identified eleven transcription factors expressed in CCAP-neurons, that have all been implicated in neuronal specification and differentiation. We have obtained mutant alleles, *UAS-dsRNAi* and UAS-wild type constructs for all of those transcription factors. Ongoing work will test the necessity of each transcription factor for all four peptide hormones. Moreover, it would be interesting to examine transcription factor expression in pre-differentiated and differentiated late CCAP-ENs. Any differences in expression would be pursued by manipulating expression of that transcription factor to test whether its presence or absence is instructive for determining the timing of late CCAP-EN differentiation. Once loss of function analysis is complete, it will be important to perform gain of function analysis to define which transcription factors are sufficient to induce ectopic CCAP-neuron peptide hormone expression.

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APPENDIX

A.1 SUPPLEMENTARY DATA CHAPTER 2.

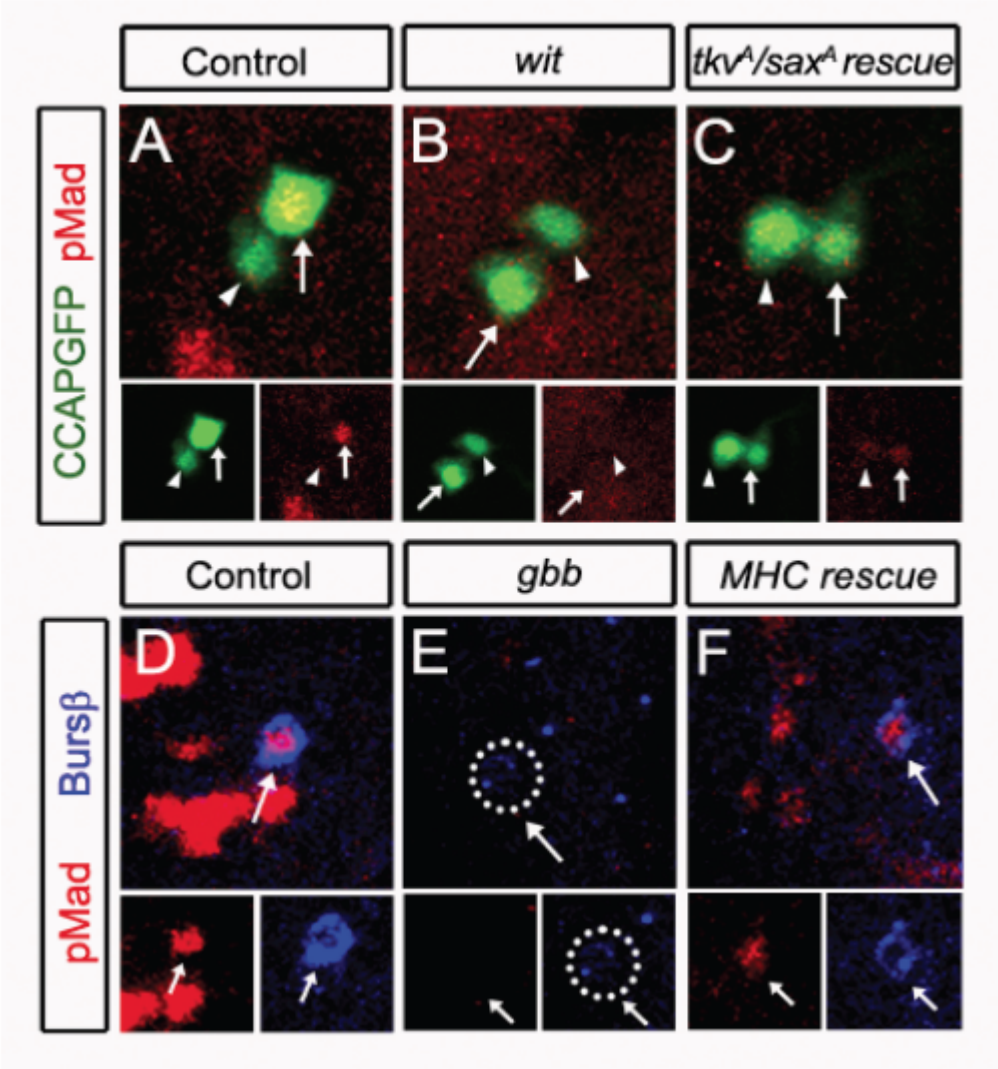

Figure 2S.1 pMad rescue in CCAP-ENs

(**A**) In L3 controls, strong pMad immunoreactivity was present in CCAP-ENs (arrow). (**B**) In *wit* mutants, pMad immunoreactivity was absent. (**C**) *Ccap-GAL4* restoration of *UAS-tkv*^{Act}, *UAS-sax*^{Act} in *wit* mutants partially rescued pMad immunoreactivity in CCAP-ENs (arrow). This contrasts with *Ccap-GAL4*/*UAS-wit* rescue of *wit* mutants, in which pMad levels were restored to control levels (see **Fig. 2.2L**). (**D**) In late L1 controls, strong pMad immunoreactivity and Bursβ expression were present in CCAP-ENs (arrow). (**E**) In *gbb* mutants, pMad immunoreactivity was absent and Bursβ expression was significantly downregulated. (**F**) In *gbb* mutants, *MHC-GAL4*^{Geneswitch} restoration of *gbb* partially rescued pMad immunoreactivity and Bursβ expression. Genotypes: (A) control (*Ccap-GAL4,UAS-nEGFP*/+;*wit*^{A12}/+); (B) *wit* (*Ccap-GAL4,UAS-nEGFP*/+;*wit*^{A12}/*wit*^{B11}); (C) *tkv*^{Act}/*sax*^{Act} rescue (*Ccap-GAL4,UAS-nEGFP*/*UAS-tkv*^{Act},*UAS-sax*^{Act};*wit*^{A12}/*wit*^{B11}); *wit*$^{-/-}$ *UAS tkv*^{Act}/*sax*^{Act} (+/*UAS-tkv*^{Act},*UAS-sax*^{Ac};*wit*^{A12}/*wit*^{B11}); (D) control (*gbb*^{1}/+;+/*MHC-GAL4*^{Geneswitch}); (E,F) *gbb*^{1}/*UAS-gbb,gbb*^{1};*UAS-gbb*/*MHC-GAL4*^{Geneswitch} minus RU486 (mutant) or plus RU486 (rescue).

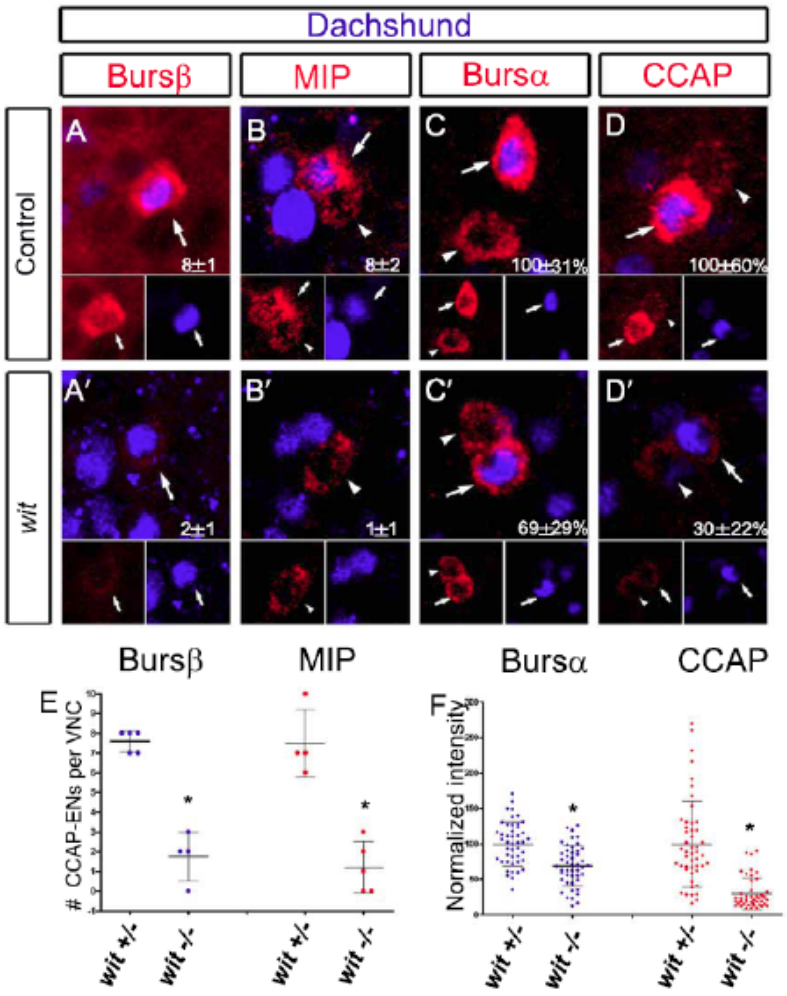

Figure 2S.2 Differential expression of *bursα*, *bursβ*, *Ccap* and *Mip* transcripts in CCAP neurons in L3 control and *wit* mutant larvae

(**A-D′**) Fluorescent in situ hybridization (FISH) for peptide hormones in pertinent genotypes. All images selected from hemisegments A1-A4. CCAP-ENs (arrows) were identified by co-expression of peptide hormone transcripts and Dac (blue, A-D). In all panels, CCAP-INs (arrowheads) were identified by expression of the peptide hormone transcript in the absence of Dac (blue, A-D′). (A-D) Representative images of *bursβ* (A, red), *Mip* (B, red), *bursα* (C, red) and *Ccap* (D; red) FISH in CCAP-EN (arrow) and CCAP-IN (arrowhead) in controls. (A′-D′) In *wit* mutants, the *bursβ*, *bursα* and *Ccap* FISH signal was reduced, whereas Mip was abolished in most CCAP-ENs. Expression in CCAP-IN was unaffected. (**E**,**F**) Scatter plots showing the number of CCAP-ENs that express Bursβ (blue dots) and Mip (red dots) (E), or the normalized intensity of Bursα (blue dots) and CCAP (red dots) in individual CCAP-ENs (F). Mean ± s.d. *, $P<0.0001$ versus pertinent controls. Genotypes: control or $wit^{+/-}$ ($wit^{A12}/+$); $wit^{-/-}$ (wit^{A12}/wit^{B11}).

Table 2S1Primer sequences for generation of *UAS-peptide* hormone and *in situ* hybridization

Gene	Function*	Sequence (59 to 39)
CCAP	Probe	5' CGCTCCTCCAATTGCTGC 3' 5' GGATTTCCCTGAGGCTGC 3'
	UAS	5' AGATCTATGAGAACGTCCATGAGGATT 3' 5' TCTAGATCATTTGCTTTCGCGCTCCTC 3'
MIP	Probe/UAS	5' AGATCTTATGGCTCACACTAAGACG 3' 5' TCTAGAATTAGTTGCTGGGCAACTG 3'
Bursb	Probe	5' GCATGTCCAGGAACTGCTCT 3' 5' TTAATAACGCCCATAGTTGG 3'
	UAS	5' AGATCTATGCATGTCCGGAACTGCTC 3' 5' CTCGAGTTAACGTGTGAAATCGCCACA 3'
Bursa	Probe	5' TTTACGCTCGCCGGGCTTCA 3' 5' ACCTGCTCCGCCACGAGAACAA 3'

*Probe, to amplify genomic or coding sequence (CDS) regions in order to generate sense and antisense single-stranded DNA probes for *Ccap*, *Mip*, *bursα* as well as the RNA probe for ***bursβ*; UAS, to amplify CDS for each peptide hormone to generate each *UAS*-transgene**

Table 2S2 Expression of Bursα, Bursβ, CCAP and Mip in CCAP-INs in *wit* mutants

Peptide hormone	*OK6* Control	*OK6 wit* $^{-/-}$
Bursα	100±32.8% n=122	106.1±34.4% n=122; P=0.3
Bursβ	100±44.0% n=102	85.6±26.6% n=194;P=0.01*
CCAP	10.3±2.0 n=16	11.1±1.9 n=15; P=0.21
MIP	7.5±2.3 n=12	7.1±1.6 n=13; P=0.6

In *wit* mutants (wit$^{-/-}$), no change in CCAP, Mip or Bursα expression was observed in CCAP-INs. Bursβ expression was subtly downregulated with marginal significance, as illustrated in the scatter plot (below), which shows the distribution of normalized intensity of Bursβ expression in control animals and wit mutants, emphasizing the limited level of downregulation despite marginal statistical significance (mean ± s.d). Genotypes: *OK6* control (*OK6-GAL4, UAS-nEGFP/+; wit^{A12}/+); OK6 wi$^{t-/-}$* (*OK6-GAL4, UAS-nEGFP/+; wit^{A12}/wit^{B11}*).

†Expression for each peptide hormone is expressed as the relative (percentage) fluorescence intensity (normalized to the mean of the pertinent control) per individual CCAP-EN (n, the number of CCAP-ENs).

‡The number of CCAP-INs per VNC that express the peptide hormone

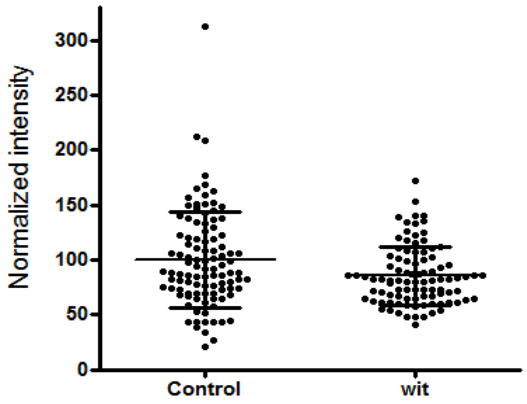

Table 2S3 Total number of type III boutons and axonal branch in controls, *wit* mutants and *wit* rescue

	Number of boutons	Total projection length (μm)
CCAP-GAL4,UAS-CD8-GFP/+; wit^{A12}/+	25.0±11.4 n=18	470.7±159.4 n=18
CCAP-GAL4,UAS-CD8-GFP/+; wit^{A12}/wit^{B11}	12.2±5.5 n=20; P=0.0009*	310.6±104 n=20; P=0.0005*
CCAP-GAL4,UAS-CD8-GFP/UAS-wit; wit^{A12}/wit^{B11}	19.9±11.8 n=21; P=0.02**, P=0.15, NSD*	572.4±137.2 n=21; P<0.0001**; P=0.02*

In *wit* mutants Type III synapses on muscle 12 had significantly fewer boutons and shorter projection length compared to control animals. Restoration of *wit* function using *CCAP-GAL4* resulted in a full rescue of bouton number and a significant expansion of projection length compared to control.

* Compared to pertinent Control. ** Compared to mutant. NSD, No Significant Difference.

A.2 SUPPLEMENTARY MATERIAL CHAPTER 3

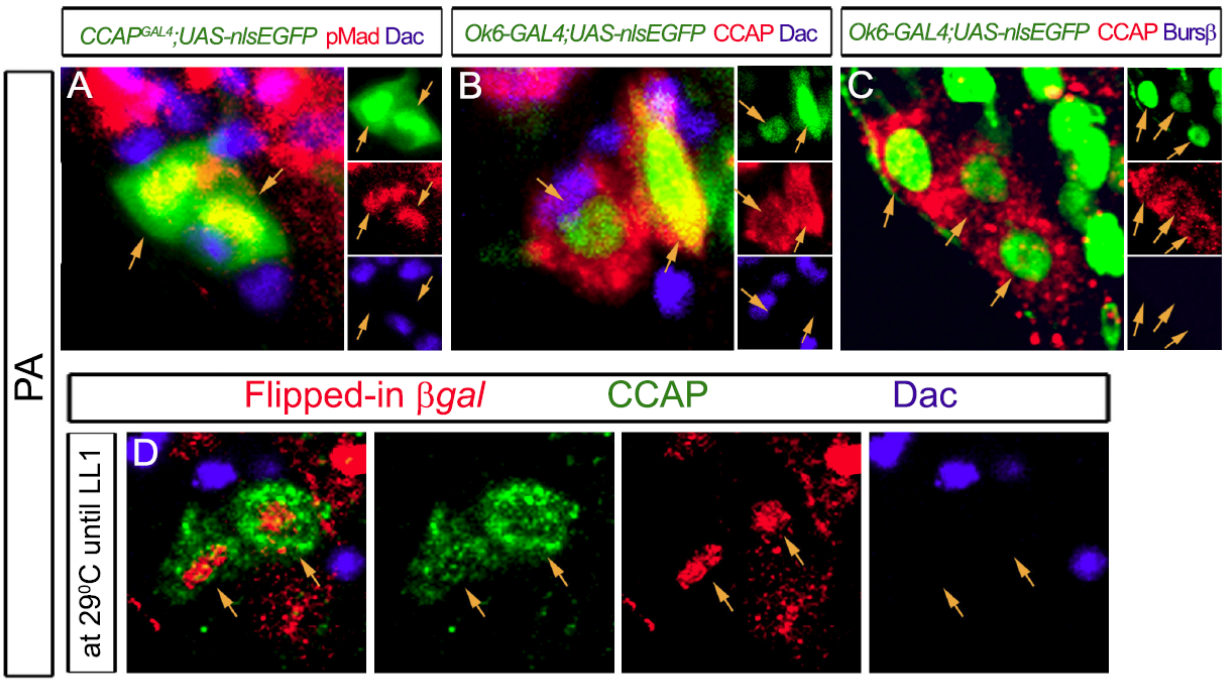

Figure 3S1. Molecular markers expressed in CCAP-PL neurons in pharate adults

(**A-C**) Triple label and fluorophore splits in pharate adults (PA). CCAP-PLs (orange arrows) can be distinguished from other CCAP-neurons by position at the posterior lateral edges of the ventral nerve cord in A8/A9 and their irregular shape, compared to the rounder CCAP-ENs and CCAP-INs. **(A)** CCAP-PLs express *CCAP-GAL4,UAS-nEGFP* (green) and anti-pMad (red) but do not express Dac. **(B)** They express *OK6-GAL4,UAS-nEGFP* (green) and the CCAP neuropeptide (red). (**C**) They do not express Bursα (shown in Fig 1 **C',D**) or Bursβ. **(D)** Using *OK6-GAL4* with TARGET and Flp/FRT systems, we raised animals from late St17 to early L2 (prior to post-embryonic neurogenesis) and switched to 18°C thereafter. In pharate adults, anti-β-Gal was observed in all CCAP-PLs (orange arrows). Thus, all CCAP-PLs were born and expressed *OK6-GAL4* between late St17 and early L2.

Genotypes. (**A**) *CCAP-GAL4,UAS-nEGFP/+*; (**B,C**) *OK6-GAL4,UAS-nEGFP/+*; (**D**) *$OK6^{GAL4}$/Act-FRT>STOP>FRT-nlsLacZ; $tubP\text{-}GAL80^{TS}$, UASnEGFP /UAS-Flp*.

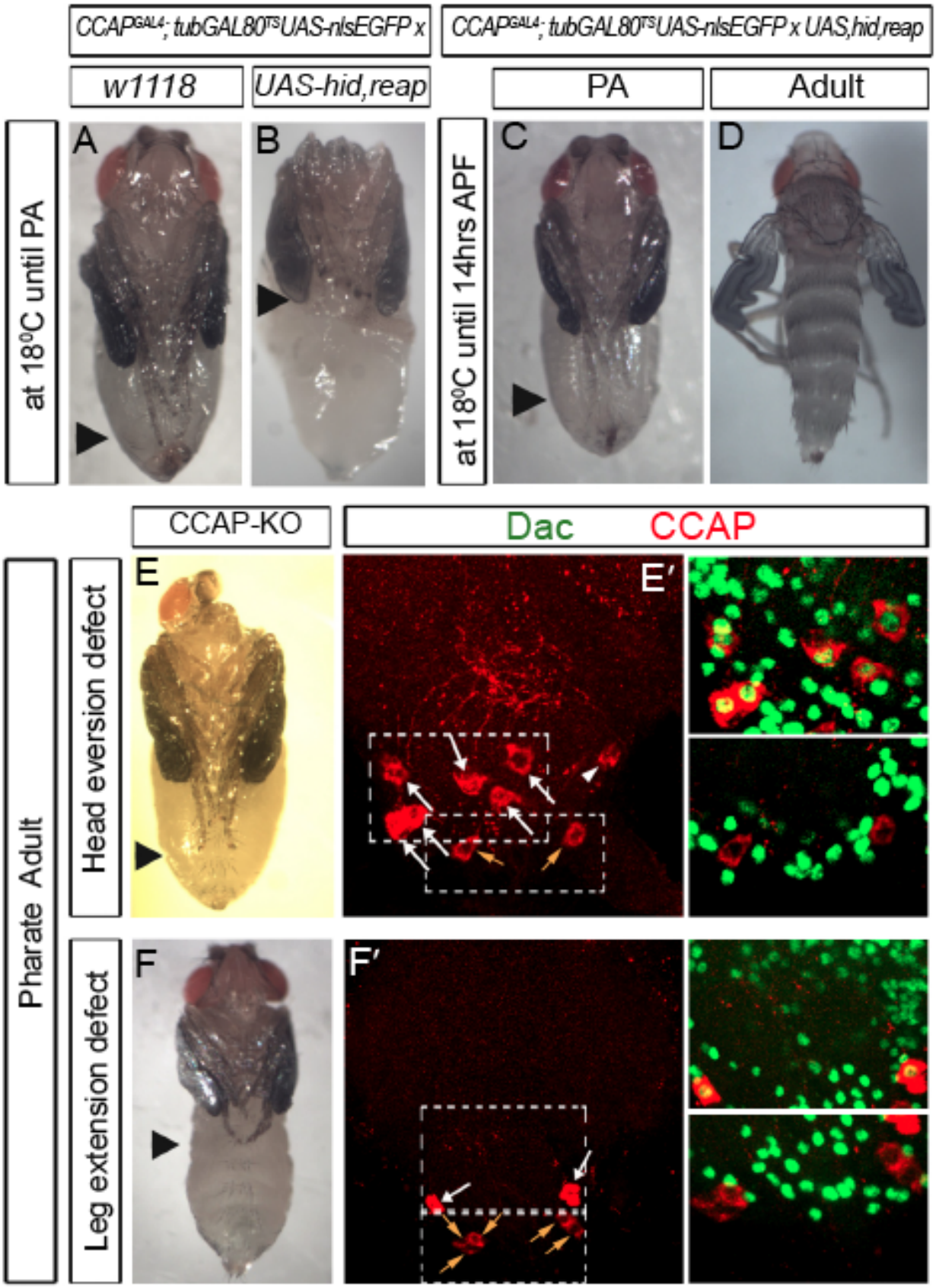
CCAPGAL4; tubGAL80TS UAS-nlsEGFP x
CCAPGAL4; tubGAL80TS UAS-nlsEGFP x UAS,hid,reap
w1118
UAS-hid,reap
PA
Adult
at 18°C until PA
at 18°C until 14hrs APF
A
B
C
D
CCAP-KO
Dac
CCAP
Pharate Adult
Head eversion defect
Leg extension defect
E
E′
F
F′

Figure 3S2. Head eversion and leg extension are directed by a different late CCAP-neuron subsets

Subset-selective ablation of late CCAP-neurons show that late CCAP-ENs direct leg extension whereas CCAP-PLs direct head eversion. **(A)** Control pharate adults (PA) had wildtype leg extension (arrowhead) and head eversion. (**B**) Ablation of all CCAP-neurons blocked leg extension and head eversion. Animals raised at 29^{o}C to PA to inactivate GAL80^{TS} so that *CCAP-GAL4* drives Hid/Reaper expression. (**C,D**) Ablation of all CCAP-neurons immediately after pupal ecdysis (at 14hr APF) resulted in wildtype leg extension and head eversion (C). 100% of these animals eclosed but their wings failed to inflate (D). (**E,F**) Switching animals from 29^{o}C to 18^{o}C after pupariation but before pupal ecdysis (to deactivate *CCAP-GAL4* and cell death) resulted in PA animals with either (E) failed head eversion but normal leg extension, or alternatively (F) normal head eversion but failed leg extension. (**E-F'**) Examination of CCAP-neuron distribution in these two sets of animals showed that all early CCAP-neurons were ablated but different subsets of late CCAP-neurons remained. (E') Head eversion defect / leg extension wildtype animals had most late CCAP-ENs (white arrows; Dac^{+ve} shown in green), but lost most CCAP-PLs (orange arrows; Dac^{-ve}) (F') Head eversion wildtype / leg extension defect animals had CCAP-PLs but lost most CCAP-ENs. **Genotypes.** (**A-A''**) +/+; *CCAP-GAL4/+; tubP-GAL80^{TS},UAS-nEGFP/+*; (**B-F'**) *UAS-hid,UAS-reaper/+* or Y; *CCAP-GAL4; tubP-GAL80^{TS},UAS-nEGFP/+* .

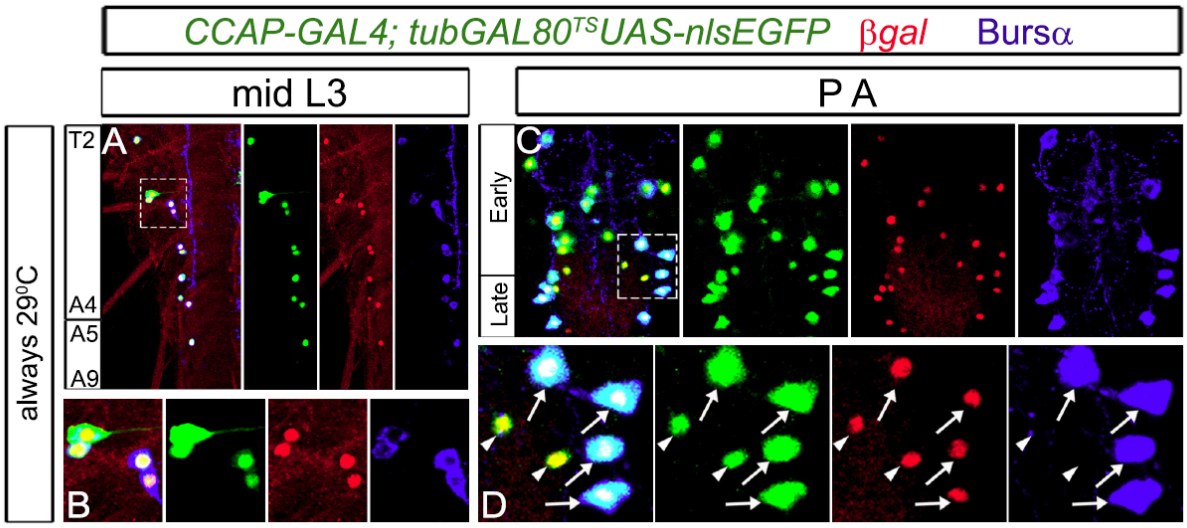

Figure 3S3. The Flp/FRT system funcitons to Flp-in β-Gal into all CCAP-neurons

Combining the TARGET and Flp/FRT systems, we permanently β-Gal-marked neurons. We maintained animals at 29°C throughout all development up to PA. **(A,B)** Mid L3 Hemi-VNC (T2-A9) including triple label and fluorophore splits. *CCAP-GAL4* (green) and anti-Bursα (blue) identify early CCAP-neurons. β-Gal (red) labeled all differentiated early CCAP-neurons but no late CCAP-neurons. (**C,D**) In pharate adults (PA), *CCAP-GAL4* (green) labeled all CCAP-neurons, but Bursα (blue) only labeled CCAP-ENs. Every CCAP-neuron showed β-Gal (red) expression. **(C)** Early and late subsets are demarcated, and both subsets express β-Gal. **(D)** Closeups of inset box from (C) showing that CCAP-ENs (arrow) express *CCAP-GAL4* (green) and Bursα (blue) whereas CCAP-INs (arrowhead) only express *CCAP-GAL4*. However, both subsets express β-Gal (red). **Genotypes.** *CCAP-GAL4 /Act-FRT>STOP>FRT-nlsLacZ; tubP-GAL80^{TS}, UAS-nEGFP /UAS-Flp*

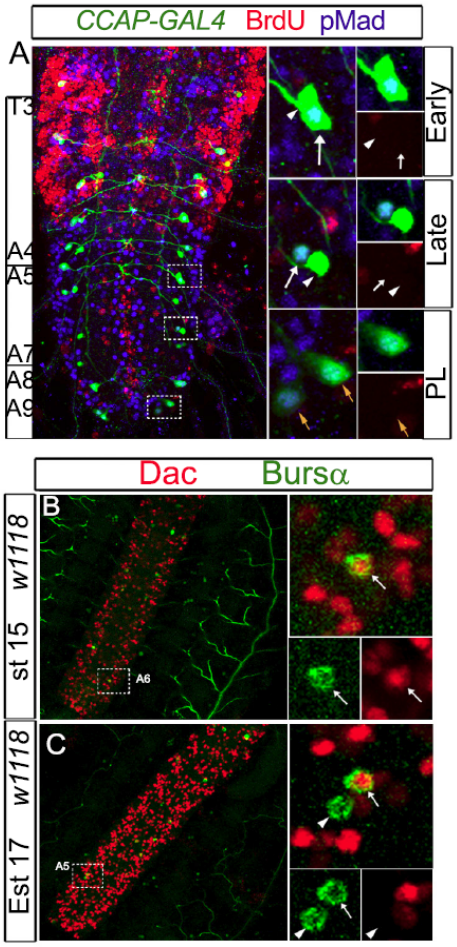

Figure 3S4. Absence of BrdU incorporation in all late CCAP-neurons and transient turn on of Bursα at embryonic Stage 15 points toward embryonic origin of late CCAP-neurons

(**A**) Animals were fed BrdU-supplemented yeast paste from early L1 until pupariation. 2hr APF pupae VNC are triple labeled (with fluorophore splits) for *CCAP-GAL4* (green), BrdU (red) and pMad (blue). BrdU labeling is extensive throughout the VNC. However, no CCAP-neuron exhibited BrdU labeling. Boxed regions are shown as closeups to show the lack of BrdU incorporation into CCAP-INs (arrowhead) and early CCAP-ENs (arrow) (Early), into late CCAP-ENs (arrow) (Late) or into CCAP-PLs (orange arrow) (PL). **(B,C**) Embryonic stage 15 (A) and early stage 17 (B) w^{1118} embryonic VNC double-labeled (with fluorophore splits) for Bursα (green) and Dac (red). Bursα-expression can be observed in a Dac^{+ve} CCAP-neuron in segments A5-A7. This is often accompanied with a Dac^{-ve} CCAP-neuron (arrow) in the same hemisegment, a CCAP-IN (arrowhead). Thus, late CCAP-ENs appear to become postmitotic and

transiently express the differentiation marker, Bursα, in St15 embryos before larval stages. **Genotypes:** (A) *CCAP-GAL4,UAS-nEGFP/+*; (B,C) w^{1118}.

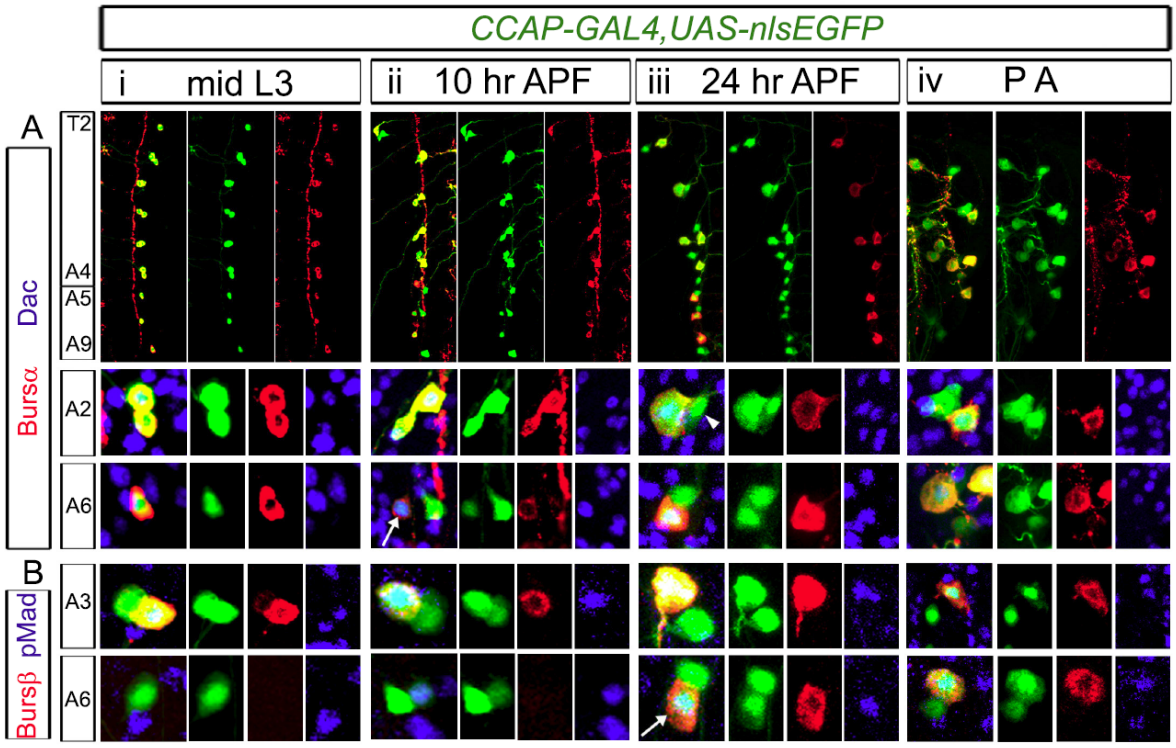

Figure 3S5. Late CCAP-neurons start to differentiate by late L3

Late CCAP-neurons started to differentiate at late L3, prior to pupal ecdysis (which is at 12hr after puparium formation; APF). Expression of Bursα (**A**) and Bursβ (**B**) in CCAP-neurons at mid L3 before late-CCAP neuron differentiation **(i)**, at 10hr APF immediately prior to pupal ecdysis **(ii)**, at 24hr APF **(iii)**, and at pharate adult (PA) **(iv). (A)** T2-A9 hemi-VNC showing *CCAP-GAL4* (green) and Bursα (red) expression in CCAP-neurons. A2 and A6 hemisegment close-ups of *CCAP-GAL4* (green), Bursα (red) and Dac (blue). (**Ai-iv;A2**) T3-A4 hemisegments had a CCAP-neuron doublet (Dac^{+ve} CCAP-EN, Dac^{-ve} CCAP-IN) from mid L3 (Ai) to PA (Aiv). Bursα expression was lost in CCAP-IN by 24hr APF (arrowhead in Aiii). (**Ai-iv;A6**) In hemisegments A5-A7, one CCAP-neuron (Dac^{-ve} CCAP-IN) was seen at mid L3. By pupal ecdysis (ii) and thereafter, a late CCAP-EN (Dac^{+ve}, $CCAP^{+ve}$, $Bursα^{+ve}$) had differentiated (arrow in Aii). (**B**) Expression of Bursβ (red) in hemisegments T3-A4 (A3) and A5-A7 (A6). Bursβ was only expressed in CCAP-ENs ($CCAP^{+ve}$, $pMad^{+ve}$) from mid L3 to PA. Bursβ expression was induced at the onset of pupal ecdysis (2hrs after the 10hrAPF timepoint shown) and was retained in all CCAP-ENs up to PA (arrowhead in Biii). **Genotypes:** *CCAP-GAL4,UAS-nlsEGFP/+*.