A *Drosophila* Gain-of-Function Screen for Candidate Genes Involved in Steroid-Dependent Neuroendocrine Cell Remodeling

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ABSTRACT

The normal functioning of neuroendocrine systems requires that many neuropeptidergic cells change, to alter transmitter identity and concentration, electrical properties, and cellular morphology in response to hormonal cues. During insect metamorphosis, a pulse of circulating steroids, ecdysteroids, governs the dramatic remodeling of larval neurons to serve adult-specific functions. To identify molecular mechanisms underlying metamorphic remodeling, we conducted a neuropeptidergic cell-targeted, gain-of-function genetic screen. We screened 6097 lines. Each line permitted Gal4-regulated transcription of flanking genes. A total of 58 lines, representing 51 loci, showed defects in neuropeptide-mediated developmental transitions (ecdysis or wing expansion) when crossed to the panneuropeptidergic Gal4 driver, *386Y-Gal4*. In a secondary screen, we found 29 loci that produced wing expansion defects when crossed to a crustacean cardioactive peptide (CCAP)/bursicon neuron-specific Gal4 driver. At least 14 loci disrupted the formation or maintenance of adult-specific CCAP/bursicon cell projections during metamorphosis. These include components of the insulin and epidermal growth factor signaling pathways, an ecdysteroid-response gene, *cabut*, and an ubiquitin-specific protease gene, *fat facets*, with known functions in neuronal development. Several additional genes, including three micro-RNA loci and two factors related to signaling by Myb-like proto-oncogenes, have not previously been implicated in steroid signaling or neuronal remodeling.

NEURONS display extensive morphological and functional changes after terminal differentiation. The resulting changes in neuronal activity shape nervous system homeostasis, seasonal and developmentally staged behavior, learning, and responses to stress, injury, and disease (BURBACH *et al.* 2001; ZITANOVA *et al.* 2001; THIERY *et al.* 2002; VIAU 2002; KARMARKAR and DAN 2006; ROMEO and McEWEN 2006; ARANCIO and CHAO 2007; NAVARRO *et al.* 2007). In recent years, exponential progress has been made toward understanding the molecular and cellular mechanisms underlying neuronal plasticity. However, the factors governing developmental remodeling in neuroendocrine systems remain poorly understood.

Metamorphosis of the insect nervous system involves extensive developmental reorganization. Differentiated

larval neurons adopt one of two fates: programmed cell death or morphological remodeling (TRUMAN 1992). The programmed cell death of many larval neurons occurs through autophagy or apoptosis (WEEKS 2003; CHOI *et al.* 2006). Neuronal remodeling involves the selective elimination of larval neurites and the outgrowth and elaboration of adult-specific projections (LEVINE and TRUMAN 1982; LEE *et al.* 2000). These events support the transformation of the insect from a vermiform larva that is devoted to feeding into an active adult with well-developed legs, wings, and sensory organs and complex reproductive behavior. Molting and metamorphosis are coordinated by two families of hormones, the juvenile hormones and the ecdysteroids (NIJHOUT 1994). During metamorphosis, ecdysteroids act cell autonomously to control neuronal cell fates (ROBINOW *et al.* 1993; LEE *et al.* 2000; BROWN *et al.* 2006) through evolutionarily conserved cellular mechanisms and signaling pathways (DRAIZEN *et al.* 1999; KINCH *et al.* 2003; WATTS *et al.* 2003; CHOI *et al.* 2006; HOOPFER *et al.* 2006).

Successful molt completion requires precise timing of ecdysis behaviors, which lead to shedding of the old cuticle. This is controlled, in part, by declining ecdysteroid levels that act through a hierarchical cascade of neu-

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ropeptides and peptide hormones to trigger the behaviors (EWER and REYNOLDS 2002). Intensive study over the past four decades has revealed many salient features of this hierarchy, although some components of the system remain to be identified. In the current model (EWER and REYNOLDS 2002), one of the first steps is activation of the endocrine Inka cells, which are located peripherally on the tracheae. In *Drosophila*, the Inka cells produce two related peptide hormones, referred to collectively as ecdysis-triggering hormone (ETH). ETH stimulates the secretion of additional peptide hormones, including eclosion hormone (EH) and crustacean cardioactive peptide (CCAP). These and other neuropeptides contribute to the control of ecdysis behaviors (CLARK *et al.* 2004; KIM *et al.* 2006). After ecdysis, some of the CCAP neurons are thought to secrete CCAP and additional neuropeptides to control postecdysis behaviors. These include bursicon, a heterodimeric neuropeptide hormone that controls wing expansion behaviors and cuticular sclerotization shortly after adult ecdysis.

The CCAP/bursicon neurons undergo substantial remodeling during the pupal stage (LUAN *et al.* 2006). These changes likely underlie some of the differences following metamorphosis in the timing, pattern, and function of ecdysis and postecdysis behaviors. Many other neuropeptidergic cells with known or potential roles in the control of molting-related behaviors also undergo metamorphic remodeling (*e.g.*, RIDDIFORD *et al.* 1994; SCHUBIGER *et al.* 1998). These changes are accessible for relatively high-throughput genetic screens. Although ecdysis and postecdysis behaviors are generally completed within a few minutes, the targeted ablation of the *Drosophila* CCAP/bursicon and EH neurons results in a variety of easily observable phenotypes that can be scored days later. These include failure to evert the adult head at pupal ecdysis and failure to expand the wings and sclerotize the adult cuticle (MCNABB *et al.* 1997; PARK *et al.* 2003). Similar phenotypes are produced through the targeted manipulation of cell signaling within these neurons (CHERBAS *et al.* 2003; PARK *et al.* 2003; HODGE *et al.* 2005; LUAN *et al.* 2006).

Drosophila genetic mosaic methods provide powerful tools for the inhibition or stimulation of gene function in small numbers of neurons (down to the level of single cells) and at specific stages in development (LEE and LUO 1999; MCGUIRE *et al.* 2004). These tools allow the experimental manipulation of signaling pathways involved in metamorphic remodeling within the complex brain, where cells display the impact of these changes within the context of hormonal signals, metabolic and other microenvironmental cues, and other cellular interactions. Coupled with our detailed understanding of the neuroendocrine control of metamorphosis and molting behaviors, this system provides unique opportunities to perform unbiased genetic screens for novel regulators of neuronal remodeling. However, genes that regulate nerve cell remodeling during metamorphosis

are likely to also regulate the embryonic or larval development of neurons (temporal pleiotropy) or the development of other tissues (spatial pleiotropy). If these other functions are essential to the survival of embryos or larvae, then they will preclude the observation of loss-of-function (LOF) mutant phenotypes involving the disruption of neuropeptidergic cell metamorphosis. Gain-of-function (GOF) screening may overcome these problems of spatial and temporal pleiotropy and can also reveal gene functions even when other genes have redundant functions (RØRTH 1996; RØRTH *et al.* 1998). As a proof of principle, GOF screens in several other well-studied *Drosophila* developmental models have identified many genes with previously confirmed roles (RØRTH 1996; RØRTH *et al.* 1998; ABDELILAH-SEYFRIED *et al.* 2000; KRAUT *et al.* 2001; PENA-RANGEL *et al.* 2002; TSENG and HARIHARAN 2002; MCGOVERN *et al.* 2003). GOF screens also have resulted in the identification of several novel and important developmental regulators, thus confirming the utility of this approach for gene discovery (BRENNECKE *et al.* 2003; SHERWOOD *et al.* 2004; TELEMAN *et al.* 2005, 2006).

Here, we performed a GOF screen to identify candidate regulators of ecdysteroid-dependent metamorphosis of neuropeptidergic cells. We first used the Gal4/upstream activating sequence (UAS) system to direct expression of several known cell-signaling regulatory molecules to three different populations of neuropeptidergic cells. These experiments established the feasibility of this approach by demonstrating our ability to detect ecdysis and wing expansion defects in the progeny. We then used this method to perform a systematic GOF screen of 6097 lines that provided neuropeptidergic cell-targeted activation of randomly selected loci in the *Drosophila* genome. These experiments revealed at least 14 loci with putative functions in the formation or maintenance of adult-specific neurite projections during metamorphosis. Our screen also revealed the existence of additional, as yet unidentified, neuropeptidergic cells with critical roles in the signaling hierarchy controlling ecdysis and wing expansion.

MATERIALS AND METHODS

Stocks: Flies (*Drosophila melanogaster*) were cultured on standard cornmeal–yeast–agar media at 22°–25°, and test crosses were performed at 29° unless otherwise noted. *EP* lines with insertions on the second and third chromosomes (RØRTH 1996; RØRTH *et al.* 1998) were obtained from the Szeged *Drosophila* Stock Center. *EP* lines with insertions on the X chromosome and *EY*, *WH*, and *XP* lines (BELLEN *et al.* 2004) with insertions on chromosomes X–4 were obtained from the Bloomington *Drosophila* Stock Center. The Gal4 drivers used were *EH-Gal4* (*w*^{*}; *P*[*GAL4-Eh.2.4*]/*C21*; FBti0012534) (MCNABB *et al.* 1997), *c929-Gal4* (*w*^{*}; *P*[*GawB*]/*cr*⁹²⁹; FBti0004282) (O'BRIEN and TAGHERT 1998), *386Y-Gal4* (*w*^{*}; *P*[*GAL4*]/*386Y*; FBti0020938) (BANTIGNIES *et al.* 2000), and *CCAP-Gal4* (*y*^{*} *w*^{*}; *P*[*Ccap-GAL4.P*]/*16*; FBti0037998) (PARK *et al.* 2003). Other lines were obtained from the Bloomington *Drosophila* Stock Center

or were kindly provided by individual labs (supplemental Table 1 at <http://www.genetics.org/supplemental/>).

Insertion-site analysis: Flanking DNA sequences for the target lines used in this screen were previously determined [EP and EY lines (ABDELILAH-SEYFRIED *et al.* 2000; BELLEN *et al.* 2004) and XP and WH lines (THIBAUT *et al.* 2004)]. For each target line obtained through the GOF screening, we used the University of California, Santa Cruz (KAROLCHIK *et al.* 2003), and FlyBase (GRUMBLING *et al.* 2006) Genome Browsers and the BLASTN user service at the National Center for Biotechnology Information to identify transcripts near the insertion. We defined the putative target gene(s) as the first locus (loci) within 30 kb of the insertion site and in the same orientation (GOF) as the run-off transcript derived from the UAS sites in the target element. We also identified loci that were in reverse orientation, within 5 kb of the insertion, and located closer to the insertion than any transcripts in the forward orientation, as potential mediators of LOF phenotypes (*cf.* ABDELILAH-SEYFRIED *et al.* 2000).

We used three criteria—phenocopy with UAS transgenes, induction of elevated immunostaining, or induction of *in situ* hybridization signals—to independently validate the identification of the misexpressed genes for selected GOF lines. The identifications of 9 loci have been validated, including 7 of the 17 loci included in our analysis of CCAP/bursicon cellular phenotypes (see supplemental Results and Discussion at <http://www.genetics.org/supplemental/>): *cabut* (*cbt*), *fat facets* (*faf*), *forkhead box sub-group O* (*foxo*), *miR-310–miR-313*, *miR-276a*, *miR-279*, *Myb-interacting protein 120* (*mip120*), *pointed* (*pnt*), and *split ends* (*spen*).

Immunocytochemistry and quantification: Immunostaining was performed on central nervous system (CNS) or whole-animal fillet preparations obtained from wandering larvae, prepupae at the indicated times after puparium formation (APF), staged pupae (BAINBRIDGE and BOWNES 1981), or adults at the indicated times after eclosion. After dissection in calcium-free saline [182 mM KCl, 46 mM NaCl, 2.3 mM MgCl₂·6H₂O, 10 mM 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris), pH 7.2], tissues were fixed for 1 hr at room temperature (RT) in either 4% paraformaldehyde (PFA) or 4% paraformaldehyde/7% picric acid (PFA/PA), and immunostaining was performed as described (HEWES *et al.* 2003, 2006). We used antisera directed against the following proteins: CCAP (1:4000, PFA/PA) (PARK *et al.* 2003), Bursicon α -subunit (1:5000, PFA/PA) (LUAN *et al.* 2006), green fluorescent protein (GFP) (1:500, PFA) (Invitrogen, Carlsbad, CA), Split ends (SPEN) (1:50, PFA) (CHEN and REBAY 2000), and Myb-interacting protein 120 (MIP120) (1:1000, PFA) (LEWIS *et al.* 2004). Confocal z-series projections were obtained using an Olympus (Center Valley, PA) Fluoview FV500 microscope. Many of the grayscale images were inverted in Adobe (San Jose, CA) Photoshop for better visualization of fine cellular processes. Images of external structures were obtained on an Olympus SZX12 stereomicroscope with a SPOT RT camera and software (Diagnostic Instruments, Sterling Heights, MI). Photomontages of the images were obtained using Adobe Photoshop. Test and control preparations, and preparations in each developmental time series, were stained and imaged in parallel.

For quantification of the extent of neurite pruning and outgrowth during metamorphosis and of bursicon secretion in adult animals, we used the threshold function in Adobe Photoshop (with the same threshold for all images) to convert the background to white and all remaining pixels (neurites and somata) to black. Somata and any obvious artifacts were manually cut from the image, and then we obtained a count of the black pixels. For time points during which pruning and outgrowth overlapped, the extent of each was obtained by manually cutting away portions of the arbor and then recount-

ing the number of black pixels. We reported variances as standard errors (for means) or interquartile ranges (IQR, distance between the 75th percentile and the 25th percentile). Statistics were performed using NCSS-2001 software (NCSS, Kaysville, UT).

In situ hybridization with locked nucleic acid probes: We performed *in situ* hybridization using a protocol modified from LI and CARTHEW (2005) with digoxigenin (DIG)-labeled locked nucleic acid (LNA) probes (Exiqon, Woburn, MA). The probes and the modified protocol are described in the supplemental Materials and Methods at <http://www.genetics.org/supplemental/>. We obtained near-infrared fluorescent images of the insoluble purple precipitate (McCAULEY and BRONNER-FRASER 2006; TRINH *et al.* 2007), using a Zeiss Axio Imager Z1 system with an Hg arc lamp source, a 645- to 685-nm excitation band pass filter, and a 760-nm long pass emission filter. We also captured weak autofluorescence of the tissue with the fluorescein filters.

Expression patterns of the Gal4 drivers: We used four Gal4 lines—*EH-Gal4*, *CCAP-Gal4*, *c929-Gal4* [*dimmed-Gal4* (HEWES *et al.* 2003)], and *386Y-Gal4*—to direct GOF element expression to peptidergic cells. These lines were chosen to target different populations of cells, some of which are known to control key aspects of ecdysis and wing expansion behaviors. Reporter gene expression in the *EH-Gal4* line is limited to just two CNS neurons, the ventromedial EH neurons (McNABB *et al.* 1997). The *CCAP-Gal4* driver is expressed specifically in ~35 pairs of neurons in the brain and ventral nerve cord (VNC) that produce CCAP and bursicon (PARK *et al.* 2003; DEWEY *et al.* 2004; LUO *et al.* 2005). The *c929-Gal4* driver is expressed in several peptidergic cell types, including 100–200 heterogeneous CNS neurons, intrinsic endocrine cells in the corpora cardiaca, the endocrine Inka cells, midgut cells, and peripheral nervous system (PNS) neurons. *c929-Gal4* also drives transgene expression in scattered locations in other tissues, including fat body, epithelial cells, and salivary glands (HEWES *et al.* 2003). The *386Y-Gal4* element drives transgene expression in numerous CNS peptidergic neurons and in many peripheral endocrine cells, including the Inka cells (BANTIGNIES *et al.* 2000; TAGHERT *et al.* 2001). This driver produced the largest number of Gal4-positive neurons and secretory cells of the three lines used for this screen. All four lines yield strong transgene expression in peptidergic cells beginning in late embryos or early larvae and continuing through the adult stage (McNABB *et al.* 1997; BANTIGNIES *et al.* 2000; TAGHERT *et al.* 2001; HEWES *et al.* 2003; PARK *et al.* 2003; LUAN *et al.* 2006).

RESULTS

Overview of the GOF screen: We conducted a modular GOF screen in which we misexpressed native *Drosophila* genes in neuropeptidergic cells and examined the effects on neuropeptide-mediated developmental transitions. The screen was performed in three phases. In phase I, we examined whether gene GOF in these Gal4 patterns could produce defects in molting-related behaviors by crossing *EH-Gal4*, *c929-Gal4*, and *386Y-Gal4* to a collection of UAS lines controlling expression of cell-signaling proteins. These included wild-type, dominant-negative, or constitutively active factors involved in cAMP signaling [*dunce* (*dnc*), *Cyclic-AMP response element binding protein B* at 17A (*CrebB-17A*), *Jun-related antigen* (*Jra*), *kayak* (*kay*)], Ca²⁺ signaling [*Calmodulin*

(*Cam*), ecdysteroid action [*Ecdysone receptor (EcR)*], endocytosis [*shibire (shi)*], and electrical excitability [*Open rectifier K⁺ channel 1 (Ork1)*] (supplemental Table 1). Each of the *Gal4* drivers produced molting or metamorphosis defects in combination with multiple *UAS* lines (supplemental Table 2 at <http://www.genetics.org/supplemental/>). The mutant phenotypes (Figure 1) included (1) larval lethality, often associated with a failure to properly shed the larval mouthparts (HEWES *et al.* 2000), (2) retention of larval characteristics following pupariation, (3) pupal lethality associated with a failure to properly evert the adult head and to fully extend the legs and wings (head eversion defects) (Figure 1, C and D), (4) late pupal lethality associated with the completion of adult development [through stage P15(i) (BAINBRIDGE and BOWNES 1981)] and failure to eclose (pharate adult lethality), and (5) adults that displayed defective tanning of the cuticle and that partially or completely failed to expand their wings (Figure 1B). Similar phenotypes have previously been reported following expression of wild-type or dominant-negative constructs with all four of the above *Gal4* drivers (McNABB *et al.* 1997; BANTIGNIES *et al.* 2000; CHERBAS *et al.* 2003; CLARK *et al.* 2004; DEWEY *et al.* 2004; HODGE *et al.* 2005; KIM *et al.* 2006; LUAN *et al.* 2006).

The behaviors leading to head eversion, adult eclosion, and adult wing expansion all occur during brief (<1 hr) periods (BAKER and TRUMAN 2002; PARK *et al.* 2003). However, the disruption of any of these events leads to morphological defects that can be easily scored days later by visual examination of culture vials. Therefore, we used these three events to detect genetic interactions in phases II and III of the screen. In phase II, we crossed *EH-Gal4*, *c929-Gal4*, and *386Y-Gal4* to a collection of 1808 *E*Plines, each with an insertion on the second or third chromosome. We obtained 16 lines that displayed head eversion, adult eclosion, or wing expansion defects with the *386Y-Gal4* driver. In contrast, the *c929-Gal4* driver yielded 2 lines (both of which also interacted with *386Y-Gal4*), and the *EH-Gal4* driver produced no hits in this phase of the screen (see supplemental Results and Discussion). Therefore, in phase III of the screen, we crossed *386Y-Gal4* only to a collection of 202 *EP* lines with insertions on the X chromosome and an additional set of 4087 *EY*, *WH*, and *XP* lines with insertions on the X and all three autosomes. With the exception of *XP(3)d02595* (see below), lines that did not produce viable prepupae after two attempts were discarded and were not included in the above counts; we did not attempt to recover lines that may have produced mutant phenotypes that were restricted to larval ecdysis. In total (phases II and III combined), we obtained 57 of 6097 lines (0.93%, representing 50 independent loci) that produced defects in head eversion, adult eclosion, or wing expansion in the progeny when crossed to *386Y-Gal4*. One additional line (and locus), *XP(3)d02595*, produced larval lethality associ-

FIGURE 1.—Head eversion and wing expansion phenotypes produced by gene GOF in peptidergic neurons. (A) Oregon R (wild-type) adult female. (B) Examples of $y, w; CCAP-Gal4/EY(2)04392$ females with normal wings (normal), partially expanded wings (PEW), and unexpanded wings (UEW). The PEW and UEW phenotypes were scored as described (LUAN *et al.* 2006). Arrows indicate folds due to incomplete wing expansion in the PEW animal. (C and D) Dorsal (C) and ventral (D) views of an Oregon R (wild-type) pupa and two $y, w/w; 386Y-Gal4/EY(3)10546$ pupae displaying weak and strong microcephalic (mc) phenotypes (*cf.* HADORN and GLOOR 1943; HEWES *et al.* 2000). Cryptocephalic pupae are very similar to the strong microcephalic pupae, except that the head structures are found entirely within the thorax (HADORN and GLOOR 1943; CHADFIELD and SPARROW 1985; HEWES *et al.* 2000; PARK *et al.* 2003). The pronged bars in C indicate the anterior edges of the head and thorax and the posterior edge of the thorax. The solid and dashed lines in D indicate the posterior edges of the legs and wings, respectively. Asterisks, pupal abdomen lacking external bristles; b, posterior gas bubble due to failed anterior translocation of the gas bubble during head eversion; D, dorsal thorax dimpled; N, tanning incomplete; P, ptilinum permanently soft and partially extended; S, scutellum wrinkled and scutellar bristles crossed and directed toward anterior; T, darkened cuticle in shape of trident on dorsal thorax; X, nonglossy cuticle surface.

ated with defects in larval ecdysis. Because each of these mutant phenotypes occurs naturally at low frequencies in wild-type stocks, we scored crosses as hits in the screen only when the defects occurred in at least 10% of the pupae or adults. These 57 lines, plus *XP(3)d02595*, were then crossed to two drivers, *c929-Gal4* and *CCAP-Gal4*, that produce *Gal4* expression in subsets of the *386Y-Gal4* pattern. Only 14% (8 of 58) of the lines produced phenotypes when crossed to *c929-Gal4*, while 59% (34 of 58) of the lines did so with *CCAP-Gal4* (Table 1). Supplemental Table 3 at <http://www.genetics.org/supplemental/> lists the lines obtained in phases II and III of the screen, the distance and orientation of each insertion with respect to the reference sequence land-

TABLE 1

Lines with head eversion, pharate adult lethal, or wing expansion phenotypes with each Gal4 driver

Symbol	Transposon	Lines ^a	386Y-Gal4 phenotypes				c929-Gal4 phenotypes				CCAP-Gal4 phenotypes			
			HE	WE	P	Total (%) ^b	HE	WE	P	Total (%) ^b	HE	WE	P	Total (%) ^b
EP	P{EP}	2010	8	13	5	16 (0.8)	1			1 (0.05)	3	12		12 (0.6)
EY	P{EPgy2}	3044	9	16	12	31 (1.0)	2	2	1	4 (0.1)	6	12	1	16 (0.5)
WH	PBac{WH}	829	1			1 (0.1)	1			1 (0.1)				
XP	P{XP}	214	6	4	2	9 (4.2)	2			2 (0.9)	2	6	1	6 (2.8)
Total		6097	24	33	19	57	6	2	1	8	11	30	2	34

Only phenotypes that were visible in at least 5% of the progeny of a cross with *c929-Gal4* or *CCAP-Gal4* were counted. For *386Y-Gal4*, the cutoff was 10% (see RESULTS). HE, head eversion defective; P, pharate adult lethal; WE, wing expansion defective.

^aTotal number of lines screened that produced pupae when crossed to *386Y-Gal4*.

^bTotal lines displaying head eversion, pharate adult lethal, or wing expansion phenotypes.

mark, the genes targeted by these insertions, and the mutant phenotypes observed with each Gal4 driver.

The frequency of gene hits obtained in the screen was dependent upon the class of response element used. The rank order of effectiveness in generating phenotypes was *XP* > *EY* ≈ *EP* > *WH* (Table 1). These differences likely reflect multiple factors, including the difference in insertion-site distributions obtained with *PiggyBac* (*WH*) vs. *P* (*EP*, *EY*, and *XP*) elements (BELLEN *et al.* 2004) and the bidirectional (*XP*) vs. unidirectional (*EP*, *EY*, and *WH*) orientation of transcription off of the different drivers. However, the frequencies of hits obtained also may have been skewed by the preselection of lines deposited into the stock centers (BELLEN *et al.* 2004; THIBAULT *et al.* 2004), and our results provide only a rough estimate of the relative effectiveness of these different elements in the screen.

Most of the insertions (52 of the 55 in regions with known transcripts) were located within +1.0 kb to −7.0 kb of a confirmed transcriptional start site (EST or cDNA) or intron splice acceptor site (Figure 2). Thus, in most cases, the affected transcripts appear to be located within 7 kb of the insertion. In three lines [*EY(3)00681*, *EY(3)10546*, and *EY(3)13010*], the GOF phenotype may result from transcription over longer distances (approaching 25 kb). We obtained indirect support of this hypothesis through identification of two insertions, *EP(3)3523* and *EY(3)13010*, that are both upstream of the *miR-276a* locus. *EY(3)13010* is located ~23 kb farther away (supplemental Table 3). Both insertions led to pharate adult lethality when crossed to *386Y-Gal4*, but only *EP(3)3523* produced wing expansion defects when crossed to *CCAP-Gal4*. Therefore, while both insertions may misexpress the same locus, the closer insertion appeared to produce a stronger gain-of-function phenotype, presumably through more efficient transcription of the nearer target. However, we cannot exclude the possibility that *EY(3)13010* produced a similar phenotype through GOF of a second, unannotated gene.

Metamorphosis of the CCAP/bursicon neurons: As a basis for determining the cellular consequences of

neuropeptidergic cell overexpression of the genes obtained in the screen, we first characterized the morphology of control CCAP/bursicon neurons in larvae and in pupae at various stages of metamorphosis. We labeled the cells with either mCD8::GFP, a membrane-linked fusion protein that provides excellent visualization of fine cellular processes (visualized directly or through anti-GFP immunostaining) (LEE and LUO 1999), or antisera recognizing CCAP and the bursicon α -subunit (hereafter referred to as bursicon). Consistent with prior observations (DEWEY *et al.* 2004; LUO *et al.* 2005), in wandering third-instar larvae, most elements of the CCAP/bursicon cell pattern were visible following anti-bursicon immunostaining (Figure 3A). This generally produced a much stronger signal than anti-CCAP immunostaining (Figure 3B). However, the MP neurons and their processes in the brain and ventral nerve cord (MPA and MLT; Figure 3B) were not stained with the anti-bursicon antiserum, although they were strongly CCAP immunoreactive (*cf.* DEWEY *et al.* 2004). With the exception of the brain DP neurons (which were weakly bursicon positive and CCAP negative), most of the CCAP- and bursicon-positive somata and neurites were also clearly visible after labeling with mCD8::GFP under control of the *CCAP-Gal4* driver (Figure 3C). Some of the finer processes were more intensely labeled with the anti-neuropeptide antisera than with mCD8::GFP (*e.g.*, MPA in Figure 3, B and C), presumably due to the concentration of secretory granules in portions of the arbor.

During metamorphosis, the CCAP/bursicon cells underwent substantial remodeling, resulting in an adult pattern of neuritic projections that was markedly different from the larval pattern (Figure 3D) (LUAN *et al.* 2006). To track the fate of individual larval neurons and portions of the neurite arbor, we examined anti-bursicon and anti-GFP immunostaining on *CCAP-Gal4*, *UAS-mCD8::GFP* animals at various stages during metamorphosis: at 3-hr intervals for the first 24 hr APF, at 6-hr intervals for the period between 24 and 60 hr APF, and at 72 hr APF (Figure 4 and supplemental Figure 1 at <http://www.genetics.org/supplemental/>). We could fol-

FIGURE 2.—Scatter plot of target element insertion-site distances from the nearest promoter or exon. The distance for each target element to the 5' end of the nearest promoter (black diamonds) or exon (magenta circles) was plotted on the y-axis. Negative values represent insertions located upstream of the respective promoter or exon splice acceptor site, and positive values refer to insertions that were located 3' of these landmarks (while still 5' of significant portions of the transcript). Only the first locus within 30 kb and in the same orientation as the direction of Gal4-directed transcription off of each *EP*, *EY*, or *WH* element (unidirectional) or *XP* element (bidirectional) was included, and the distances to putative LOF (antisense) transcripts (supplemental Table 3) are not shown. Insertions within the predicted coding sequence (CDS) of a transcript, and insertions located in introns located downstream of an exon containing CDS, are indicated with open symbols.

low the neurons throughout metamorphosis—most of the larval CCAP/bursicon cells were retained—and we did not observe the development of new cells with this marker during the 72-hr period APF. We first detected the loss of bursicon/GFP immunoreactive neurites in the thoracic and abdominal ganglia at 3 hr APF. The peak amount of pruning occurred from 12 to 15 hr APF, after which the disappearance of larval neurites continued at a slower rate until ~30 hr APF. The first appearance of new, adult-specific projections occurred simultaneously with the latter stages of larval neurite pruning, and peak outgrowth occurred at 36–42 hr APF in the abdominal ganglia and at 48–54 hr APF in the thoracic ganglia. Outgrowth was essentially complete at 60 hr APF throughout the VNC.

In addition to the pruning back of larval neurites and outgrowth of new, adult-specific neurites, there were three other notable changes in the CCAP/bursicon cell

FIGURE 3.—Staining patterns and morphologies of the CCAP/bursicon neurons. (A) Anti-bursicon (BURS) and (B) anti-CCAP (CCAP) immunostaining in wandering third-instar larval CNS (*CCAP-Gal4/+*). Anti-bursicon immunostaining was also observed in a cluster of neurites located over the corpora cardiaca (not shown). (C) mCD8::GFP (*CD8::GFP*) fluorescence in a wandering third-instar CNS (*CCAP-Gal4, UAS-mCD8::GFP/+*). (D) Anti-bursicon immunostaining in a stage P14 pharate adult CNS (*CCAP-Gal4/+*). A midline protocerebral brain arbor (MPB) was also bursicon immunoreactive (not shown, see Figure 5C). Abbreviations: AA, abdominal arbor; B_{AG}^a , eight neurons located toward the anterior of the abdominal ganglia; B_{AG}^p , six neurons located in the posterior abdominal ganglia; DP, dorsal protocerebral neuron (weakly bursicon positive and CCAP negative); CTA, circum-neuropilar (ventral) thoracic arbor; DTA, dorsal thoracic arbor; LB(S, T1–3, A1–A7), lateral branch (leading from LLT to MA) in the subesophageal region and segments T1–T3 and A1–A7; LLT, lateral longitudinal tract; LSE(1–3), lateral subesophageal neurons; MA(S, T1–3, A1–A7), midline arbor in the indicated segments; MLT, midline longitudinal tract; MP, midline protocerebral neurons; MPA, midline protocerebral arbor; PA, protocerebral arbor; PAA, posterior abdominal arbor; SA, subesophageal arbor; TA, tritocerebral arbor; VA, ventral abdominal neuron. Bar, 50 μ m.

FIGURE 4.—Remodeling of the CCAP/bursicon neurons during metamorphosis. (A) Anti-bursicon immunostaining of the ventral nerve cord (VNC) at 0, 12, 36, and 60 hr after puparium formation (APF) ($n = 6$ –19). Additional time points are shown in supplemental Figure 1. (B) mCD8::GFP fluorescence in the same preparations as in A (*CCAP-Gal4, UAS-mCD8::GFP/+*). (C) Time courses for pruning and outgrowth of the neuritic arborizations in the thoracic and abdominal ganglia. The relative extent of pruning and outgrowth (indicated by the relative height, along the y-axis, of the horizontal shapes) was quantified for each of the time points represented with tick marks on the x-axis. The images in A and B are representative of the changes at the selected times. Arrows, thoracic neurites that were pruned back by the next stage shown; arrowheads, new adult-specific thoracic neurites; double-feathered arrows, abdominal neurites that were pruned back by the next stage shown. Bar, 50 μ m.

pattern in the CNS during metamorphosis. First, the somata in abdominal segments A1–A7 more than doubled in diameter (Figure 4) and later became multiangular in shape (not shown). Their orderly segmental arrangement was lost as they migrated or were pushed to new locations during the reorganization of the abdominal ganglia. Second, bursicon expression was turned off in 14 abdominal neurons. In larvae there was a pair of bursicon-immunoreactive neurons in each abdominal hemisegment. One cell in each pair was smaller and more weakly bursicon (and CCAP) immunoreactive. During the first 12–24 hr APF, these more weakly bursicon-positive neurons lost this epitope (Figure 4A and supplemental Figure 1), although they continued to express mCD8::GFP (Figure 4B). Third, a few CCAP/bursicon cells either died or turned off CCAP, bursicon, and *CCAP-Gal4* expression during metamorphosis. The most prominent example of this class of cells was the MP neurons (Figure

3), which were strongly CCAP immunoreactive in larvae but undetectable shortly after head eversion (data not shown).

In larvae, many of the CCAP/bursicon neurons in the VNC had efferent projections. We observed five pairs of unbranched axons that were weakly bursicon and CCAP immunoreactive and that projected to larval muscles 12 and 13 (HODGE *et al.* 2005). There they terminated in a few higher-order branches with numerous strongly bursicon- and CCAP-immunoreactive type III endings (supplemental Figure 2 at <http://www.genetics.org/supplemental/> and data not shown). Up to three additional pairs of axons projected out through terminal abdominal nerves. These projections did not terminate on the body wall muscles and did not branch, although strongly bursicon- and CCAP-immunoreactive varicosities were present near the distal tips (see below, Figure 6B).

To determine the fate of the CCAP/bursicon efferents during metamorphosis, we performed anti-GFP immunostaining on *CCAP-Gal4, UAS-mCD8::GFP* animals (supplemental Figure 2). At 12–24 hr APF, the larval pattern of axons was still recognizable, although the peripheral terminals retracted back toward the CNS and lost most of the larval branches and boutons. We also observed axonal fragments that may have resulted from the severing of axons during pruning (see asterisk in supplemental Figure 2). By 30–36 hr APF, the larval efferents were gone in several preparations. At this time, we saw newly forming processes that were characterized by numerous small, branching fibers near the distal tips. By 42–48 hr APF, the adult pattern of tree-like projections and branches began to form, with eight efferents projecting out a short distance through the thick medial abdominal nerve trunk and three pairs of efferents projecting out of the two or three adjacent nerves [*Ab₁Nv*, *Ab₂Nv*, and *AcNv₃* (DEMEREK 1994)]. The peripheral projections also were thicker, were more varicose, and often terminated as a mass of multiple short, poorly organized, higher-order distal branches. At 54 hr APF, the peripheral projections displayed a nearly complete adult pattern, with efferents that ramified into further, higher-order branches as they extended farther posteriorly to form a tree-like arbor. The efferents closer to the abdominal midline extended a long distance posteriorly before branching into the terminal abdominal nerves and forming large, strongly immunoreactive varicosities. The lateral efferents branched and formed strongly immunoreactive varicosities much closer to their exit points from the CNS.

Disrupted metamorphosis of the CCAP/bursicon neurons: Cell ablation experiments have shown that the CCAP/bursicon neurons are essential for head eversion behavior during pupal ecdysis as well as wing expansion and complete tanning of the adult cuticle after adult eclosion (PARK *et al.* 2003). However, for 70% of the lines that produced mutant phenotypes when *trans*-heterozygous with *CCAP-Gal4*, wing expansion defects were the only defects observed (Table 1, supplemental Table 3). Overall, 88% of the lines (*vs.* 57 and 25% with *386Y-Gal4* and *c929-Gal4*, respectively) displayed wing expansion defects either alone or together with other mutant phenotypes. In contrast, only a third of the lines produced defects in pupal ecdysis when crossed to *CCAP-Gal4*. Thus, the GOF of many of these genes may have selectively disrupted the reorganization of the CCAP/bursicon neurons during metamorphosis (whereas the cell ablations of PARK *et al.* 2003 disrupted both pupal and adult functions). To test this hypothesis, we examined anti-CCAP and anti-bursicon immunostaining and expression of *mCD8::GFP* (using direct fluorescence) in wandering third-instar larvae and in stage P14 pharate adults. To maximize our ability to detect cellular phenotypes, we focused the analysis on insertions representing the 16 loci that produced the strongest wing expansion

defects when misexpressed with *CCAP-Gal4*. In addition, we analyzed the effect of directly driving the *foxo* gene, which produced stronger wing expansion defects than the original insertion [*EY(3)11248*] upstream of the *foxo* locus. We performed most of the crosses at 29° (in parallel with crosses to the wild-type stock, Oregon R, as controls).

The results of these experiments are described in supplemental Table 4 at <http://www.genetics.org/supplemental/>. The responses of the CCAP/bursicon cells to the GOF of these 17 genes fell into at least three distinct classes: (I) gross defects in axonal pathfinding that were evident in both the larval and the adult stages, (II) selective loss of adult-specific neurites, often associated with neuronal degeneration, and (III) defective bursicon secretion in adults without obvious defects in neuron morphology. Except for the first class, the GOF of these loci (in heterozygous animals) either had no effect on the larval morphology of the CCAP/bursicon neurons or resulted in very modest cellular defects, while the adult morphology was often profoundly disrupted. For any given line the changes in cellular morphology were not a good predictor of the external phenotypes observed (and vice versa). Nevertheless, these results were consistent overall with the trend toward greater disruption of adult wing expansion and tanning than of pupal head eversion behaviors.

In addition to the above insertion-specific effects, we observed a generalized response to transgene expression in the CCAP/bursicon neurons. For most insertions, we observed 30–50% lower expression of CCAP, bursicon, and *mCD8::GFP* in most somata, central neurites, and peripheral projections at both the wandering third instar and the P14 pharate adult stages (the VA neurons, and their dorsal efferent projections, were one notable exception). We also observed this generalized reduction in immunostaining of the CCAP/bursicon neurons when we crossed *CCAP-Gal4* to *UAS-GFP* alone, even though this cross did not produce significant lethality or wing expansion/tanning defects. Therefore, this effect appeared to be a global cellular response to transgenic protein expression, and we did not examine it further. Instead, we focused our analysis on examples of the three specific classes of GOF phenotypes.

Class I—neurite pathfinding defects with *EP(2)2003 (Ptr)*: Of the 17 insertions that we examined, only one line, *EP(2)2003* [inserted upstream of *Patched-related (Ptr)*], produced widespread, gross defects in the larval CCAP/bursicon neuron pattern of dendritic and axonal projections (supplemental Table 4). Six other lines [*EP(2)2583*, *EP(2)2587*, *EP(3)3140*, *EP(3)3354*, *EP(3)3520*, and *EY(2)04392*] produced subtle defects in small portions of the CNS arbor in larvae. However, in each of these cases, the larval projection pattern was mostly preserved.

With *Ptr* GOF, portions of the normal larval pattern of CNS projections, and most of the CCAP/bursicon somata, were present. However, some somata and neurites were missing. Only 30–50% of the normal efferents and

FIGURE 5.—GOF of *Ptr* produced neurite pathfinding defects in larval CCAP/bursicon neurons. In both larvae (A and B) and pharate adults (C), misexpression of *Ptr* (A', B', and C') led to the loss of some somata and neurites (labels with dashed lines). Ectopic or mistargeted neurites were also observed (arrows). (A) mCD8::GFP (*UAS-mCD8::GFP*) expressed under the control of *CCAP-Gal4* either with (A') or without (A) *EP(2)2003*, which is inserted just 5' of the *Ptr* gene (supplemental Table 3). CNS dissections were performed at the wandering third-instar stage ($n = 8$). (B) Anti-CCAP immunostaining in a *CCAP-Gal4/EP(2)2003* wandering third-instar larval CNS (B') and a *CCAP-Gal4/+* control (B) ($n = 5$). In a second *CCAP-Gal4/EP(2)2003* CNS (B' inset), MLT was missing on one side. (C) Anti-bursicon immunostaining in the CNS from two *CCAP-Gal4/EP(2)2003* P14-stage pharate adults (C' and C'') and a *CCAP-Gal4/+* control (C) ($n = 7$). The labels are defined in the Figure 3 legend. Arrowheads, thicker neurites with larger than normal varicosities; feathered arrows, small-diameter somata. Bars: A–C, 50 μ m; B' inset, 100 μ m.

~75% of the bilateral pairs of somata in abdominal segments 1–7 (A1–A7) were visible. Many parts of the normal larval neuritic arbor were missing, often unilaterally [e.g., LB(A3) and LLT in Figure 5A' and inset in Figure 5B'], or they were substantially scaled back in size and complexity [e.g., MA(A4) in Figure 5A']. Ectopic neurites were also observed. Thus, the effects of *Ptr* GOF on the morphology of the CCAP/bursicon neurons were likely due to the disruption of neurite pathfinding. Similarly, *Ptr* produced striking defects in axonal and dendritic pathfinding during metamorphosis, leading to the bilaterally asymmetric loss of major portions of the normal neuritic arbor and the formation of ectopic neurites throughout the pharate adult CNS (supplemental Table 4, Figure 5C).

Previous work has shown that these neurons are essential for successful completion of head eversion during ecdysis from the larval to the pupal stage (PARK *et al.* 2003). Remarkably, despite the severe defects in larval CCAP/bursicon neuron morphology resulting from *Ptr* GOF, head eversion in heterozygous *CCAP-Gal4*, *EP(2)*

2003 animals occurred normally (supplemental Table 3). These results show that heterozygous *CCAP-Gal4*, *EP(2)2003* animals retained enough CCAP/bursicon, and enough larval CCAP/bursicon neuron connectivity, to allow normal functioning of the neuropeptidergic signaling hierarchy controlling pupal ecdysis behavior. In contrast, the adult function of the CCAP/bursicon neurons in controlling wing expansion was not preserved.

Class II—loss of adult-specific neurites: Of the 17 lines examined for cellular changes following GOF with the *CCAP-Gal4* driver, 14 displayed the loss of some or all adult-specific neurite projections. This group comprises *EP(2)2237*, *EP(2)2583*, *EP(3)3140*, *EP(3)3520*, *EY(X)10575*, *EY(2)04392*, *EY(2)05304*, *EY(3)00559*, *EY(3)00146*, *XP(2)d07339*, *XP(3)d00809*, *XP(3)d02595*, *XP(3)d04253*, and *UAS-foxo*, which was substituted for *EY(3)11248* (supplemental Table 4). In each case, we detected only minor morphological changes in the CCAP/bursicon cells in larvae. These changes may have been physiologically significant, particularly when the gene dosage was increased (when more lines displayed

FIGURE 6.—GOF of *cbt* prevented the outgrowth of adult-specific bursicon-immunoreactive neurites and the increase in diameter of the CCAP/bursicon cell somata during metamorphosis. (A–D) Anti-bursicon immunostaining in the CNS (A and C) and peripheral neurites (B and D). The staining was performed on tissue dissected at the wandering third-instar larval stage (A and B) and the P14 pharate adult stage (C and D) from *CCAP-Gal4/EP(2)2237* animals (A'–D') and *CCAP-Gal4/+* controls (A–D) ($n = 5–7$). Note that the morphology of the CCAP/bursicon neurons was largely unaffected following *cbt* GOF in larvae. In contrast, the pharate adult morphology of the cells was severely disrupted. (E–H) Anti-GFP immunostaining in the CNS (E and F) and peripheral neurites (G and H) at 18 hr APF (E), 30 hr APF (G), or 54 hr APF (F and H). Membrane-associated mCD8::GFP (*UAS-mGFP*) was expressed in *CCAP-Gal4/+* (E–H) and *CCAP-Gal4/EP(2)2237* (E'–H') animals. In the CNS, *cbt* GOF caused the neurite projections to remain short, thick, and with few branches (F'), similar to the appearance of control animals 36 hr earlier (E). In the periphery, the efferents displayed some initial outgrowth, but they failed to form a complete adult arbor (H'). Asterisk, fine neuritic arbor; bicolor (solid/open) arrows, nonbranching efferent projections; solid arrows, traces of remaining neurites; solid arrowheads, smaller somata; solid double-feathered arrows, strongly immunoreactive axon terminals; solid double-feathered arrowheads, weakly immunoreactive axons; open arrows, adult-specific longitudinal axon tracts (forming or completed); open arrowheads, developing neurite branches; open double-feathered arrows, persistent larval projections after muscle detachment and before pruning is completed; open double-feathered arrowheads, adult efferents near points of exit from the CNS. Bars: A and C, 50 μm ; B, D, G, and H, 100 μm ; E and F, 10 μm .

plete adult arbor (H'). Asterisk, fine neuritic arbor; bicolor (solid/open) arrows, nonbranching efferent projections; solid arrows, traces of remaining neurites; solid arrowheads, smaller somata; solid double-feathered arrows, strongly immunoreactive axon terminals; solid double-feathered arrowheads, weakly immunoreactive axons; open arrows, adult-specific longitudinal axon tracts (forming or completed); open arrowheads, developing neurite branches; open double-feathered arrows, persistent larval projections after muscle detachment and before pruning is completed; open double-feathered arrowheads, adult efferents near points of exit from the CNS. Bars: A and C, 50 μm ; B, D, G, and H, 100 μm ; E and F, 10 μm .

head eversion defects). Nevertheless, the disruption of remodeling of the CCAP/bursicon neurons during metamorphosis (or the failure to retain adult projections) was the predominant phenotype for lines that produced defects in wing expansion or ecdysis when crossed to *CCAP-Gal4*.

We examined one insertion, *EP(2)2237*, to determine whether adult-specific neurites were lost through a pruning defect, an outgrowth defect, or atrophy. *EP(2)2237* is located immediately 5' of the *cbt* C2H2-type zinc-finger transcription factor, and overexpression of a *cbt* cDNA

phenocopied *EP(2)2237* expression in the eye disc and the wing disc (S. MUNOZ-DESCALZO, personal communication). In wandering third-instar larvae, the morphology of the CCAP/bursicon neurons was largely unchanged following *cbt* GOF with *EP(2)2237* (supplemental Table 4; Figure 6, A and B), except that we observed fewer and smaller boutons at the peripheral endings. In contrast, we observed severe defects at the P14 pharate adult stage (supplemental Table 4; Figure 6, C and D), including the loss of many central neurites and efferent projections, with the remaining efferents

FIGURE 7.—GOF of *klar* resulted in specific loss of adult-specific bursicon-immunoreactive neurites and loss of six to eight CCAP/bursicon cell somata. (A and B) Anti-bursicon immunostaining in wandering third-instar (A) and P14 pharate adult (B) stage CNS from *CCAP-Gal4/EY(3)00559* animals (A' and B') and *CCAP-Gal4/+* con-

trols (A and B) ($n = 6-8$). Most elements of the CCAP/bursicon cell projection pattern were retained in the larval CNS (A'), but there was a dramatic reduction of central neurites and B_{AG} somata number in the pharate adult (B'). Arrows, traces of remaining neurites; arrowhead, atrophied soma. Bars, 50 μm .

displaying substantially fewer/shorter branches and large varicosities (Figure 6D'). In addition, we observed the loss of 1–6 of the 14 abdominal bursicon neurons [the B_{AG} cells (LUAN *et al.* 2006)]. The remaining B_{AG} somata were reduced in diameter and either intensely immunoreactive or only very weakly immunostained (Figure 6C' and data not shown).

To determine whether these defects were due to either a failure of the CCAP/bursicon neurons to remodel or cellular atrophy after remodeling, we crossed *CCAP-Gal4, UAS-mCD8::GFP* flies to *EP(2)2237* and performed anti-GFP immunostaining on whole-animal fillets and isolated CNSs at 18, 30, 42, 54, and 60 hr APF (Figure 6, E–H). In control *CCAP-Gal4, UAS-mCD8::GFP/+* pupae at 18 hr, we observed numerous thick, anteriorly and posteriorly directed projections from the LB (Figure 6E) that appeared to set down the future location of an adult-specific axon tract located $\sim 5 \mu m$ to each side of the CNS midline (Figure 6F). While these projections were also present following *cbt* GOF, they were shorter and less numerous (Figure 6E'). These differences persisted at later stages. In the controls at 30 hr, the central neurite projections were finer and much more numerous, and the abdominal arbor displayed a more adult-like pattern. At 42 hr, the neurite tracts were thicker and more varicose. By 54 hr, the adult abdominal longitudinal axon tracts were well formed, and there were many thin neurite projections extending into the neuropile (Figure 6F). In contrast, following *cbt* GOF, the new projections retained their thick, meandering, blunt-ended appearance throughout this period and were still individually discernible even at 54 hr (Figure 6F'). Although the adult abdominal longitudinal axon tracts were visible, much of the finer abdominal neuritic arbor failed to form.

cbt GOF also resulted in defects in formation of the peripheral arbor. At 18 hr ($n = 7$), the efferents in the control pupae remained long with accumulated material at the distal endings. The pattern was similar in *CCAP-Gal4, UAS-mCD8::GFP/EP(2)2237* pupae, but the efferents were finer and had less distal end material. At 30 hr ($n = 5$), these differences were retained. However,

there were fewer efferents visible in both genotypes, and many appeared to have pruned back to the CNS (Figure 6G). By 42 hr ($n = 7$), all of the larval efferents were gone, and new projections were visible extending from the CNS. In the control pupae, these projections began to acquire a more varicose appearance and terminated in a loosely associated network of finer branches, while the *cbt* GOF efferents were thinner and less varicose and terminated in fewer branches. At 54 hr ($n = 7$) and 60 hr ($n = 3$), the *cbt*-expressing efferents were short, with only a few small branches, while the control efferents formed a nearly complete adult arbor (Figure 6H). Thus, in both the CNS and the periphery, *cbt* GOF led to an arrest in the remodeling of the CCAP/bursicon neurons at an early stage of neurite outgrowth.

Among the 14 lines displaying the loss of some or all adult-specific neurites in pharate adults, there were differences in the phenotypes that suggest the presence of multiple different underlying mechanisms. In addition to *EP(2)2237*, six other insertions [*EP(2)2583*, *EY(X)10575*, *EY(3)00559*, *EY(3)11248/UAS-foxo*, *XP(2)d07339*, and *XP(3)d02595*] displayed the loss of many central neurites and efferents, which in all of the lines except *XP(2)d07339* was accompanied by a reduced number of bursicon-immunoreactive B_{AG} (and LSE) neuron somata. In some genotypes, a subset of the observed B_{AG} somata was very small and weakly immunoreactive (Figure 7), and a few appeared to be fragmented. We observed a similar reduction in soma number by examining GFP fluorescence in pupae expressing *UAS-mCD8::GFP* in parallel with the gain-of-function transgenes (data not shown). While we cannot exclude the downregulation of bursicon, CCAP, and *CCAP-Gal4* expression in the missing neurons as the cause of the apparent loss of cells, these results are consistent with cell death. Thus, neuronal degeneration often appeared to accompany, or follow, the loss of adult-specific neurites.

A second group of five lines [*EP(3)3140*, *EP(3)3520*, *EY(2)5304*, *XP(3)d00809*, and *XP(3)d04253*] displayed similar losses of adult-specific neurites and loss of a few CCAP/bursicon neuron somata when crossed to *CCAP-Gal4*. The efferent projections displayed either failure

or reduced capacity to form *en passant* varicosities and higher-order branches. *EP(3)3140*, for example, produced efferents lacking most branches and large varicosities and ending in large, strongly bursicon-immunoreactive club-shaped endings. Thus, the GOF of these loci appeared to disrupt the accumulation of secretory material at neuroendocrine release sites.

Two insertions produced other phenotypes (supplemental Table 4). *EY(2)04392* displayed a loss of adult-specific neurites that was limited to the brain, subesophageal, and thoracic arbor in the CNS. *EY(3)00146* displayed weak immunostaining or loss of many central neurites and efferents, but the number of bursicon-immunoreactive anterior B_{AG} (B_{AG}^a) somata was increased from 8 to ~18. This was not accompanied by an increase in the number of somata labeled with *UAS-mCD8::GFP* (data not shown). In wild-type animals, the number of bursicon-immunoreactive abdominal neurons decreases from 28 to 14 during metamorphosis (compare the anti-bursicon staining to the CD8::GFP signal in Figure 4). Taken together, these results show that *EY(3)00146* blocked the downregulation of bursicon expression in 14 abdominal neurons during metamorphosis.

Class III—defective bursicon secretion without gross changes in adult cell morphology: Two insertions, *EP(2)2587* and *EP(3)3354*, produced moderate to strong, highly penetrant wing expansion defects when crossed to *CCAP-Gal4* without affecting the gross morphology of the CCAP/bursicon neurons in pharate adults (supplemental Table 4). Both *EP(2)2587* and *EP(3)3354* appear to drive the expression of micro-RNAs that all belong to the same family. *EP(2)2587* [along with two other insertions obtained in the screen, *EP(2)2586* and *EP(2)2356*] is inserted directly upstream of a cluster of four micro-RNAs, *miR-310–miR-313* (supplemental Table 3). Of the four micro-RNAs in the *miR-310–miR-313* cluster, *miR-310* is the furthest downstream of the *EP(2)2587*. We confirmed the overexpression of this micro-RNA cluster in wandering-stage *CCAP-Gal4/EP(2)2587* larvae by *in situ* hybridization with LNA probes to *miR-310* (Figure 8A; $n = 8$), *miR-312*, and *miR-313* (data not shown). In *CCAP-Gal4/+* control CNS, we observed a weak stripe of expression of *miR-310*, *miR-312*, and *miR-313* near the presumptive optic lobes (data not shown).

EP(3)3354 is inserted directly upstream of the putative transcription factor, *jing interacting gene regulatory 1* (*jigr1*). There are also two nearby micro-RNAs. *miR-92a* is located ~9 kb downstream, in an intron of *jigr1*, and *miR-92b* is ~14 kb downstream, 1 kb beyond the 3' end of the *jigr1* gene. Interestingly, *miR-92a*, *miR-92b*, and *miR-310–miR-313* are closely related (ARAVIN *et al.* 2003; LEAMAN *et al.* 2005) and share many of the same predicted target mRNAs (ENRIGHT *et al.* 2003; STARK *et al.* 2003; RAJEWSKY and SOCCI 2004; GRUN *et al.* 2005). Thus, we hypothesize that *EP(2)2587* and *EP(3)3354* affect the CCAP/bursicon neurons through inhibition

of common loci. Both insertions produced similar phenotypes, although the behavioral defects elicited with *EP(2)2587* were stronger than with *EP(3)3354*.

The failure of *EP(2)2587* and *EP(3)3354* to affect the morphology of the CCAP/bursicon neurons led us to ask whether the secretion of bursicon was affected. In *Drosophila*, bursicon activity appears in the blood within the first 20 min after eclosion (BAKER and TRUMAN 2002; LUAN *et al.* 2006). In control *CCAP-Gal4/+* flies at 22°–23°, the median times until wing expansion were 23 min after eclosion (AE) in females ($n = 26$, IQR = 18) and 12 min AE in males ($n = 23$, IQR = 9). Although the sexual dimorphism in the time to wing expansion was statistically significant ($P = 0.000048$; Mann-Whitney *U*-test), all but one of the animals of either sex initiated wing expansion within 50 min AE (one female took 473 min). In the tobacco hornworm, *Manduca sexta*, the secretion of bursicon is accompanied by an ~80% depletion of the hormone from the neuroendocrine release sites in the transverse nerve (TRUMAN 1973; TAGHERT and TRUMAN 1982), and bursicon bioactivity in the hemolymph of both *M. sexta* and blowflies peaks within 1 hr of eclosion (FRAENKEL and HSIAO 1965; REYNOLDS *et al.* 1979). Thus, we expected to be able to detect bursicon secretion through an immunofluorescence assay, as we and others have done previously to detect robust secretion of CCAP, EH, and ETH (HEWES and TRUMAN 1991; EWER *et al.* 1997; McNABB *et al.* 1997; CLARK *et al.* 2004). We performed anti-bursicon immunostaining on fillet dissections of *Drosophila* adults staged at either 0 or 1 hr AE at 25°. We collected the flies without anesthesia to preclude anesthesia-induced behavioral changes. In control *CCAP-Gal4/+* flies, we observed a 73% depletion in the intensity of anti-bursicon immunostaining over this period [Figure 8, B (top) and C]. In wild-type animals, there was a concomitant depletion of CCAP and PHM immunoreactivity at this time (data not shown). Taken together with the earlier bioassay data (BAKER and TRUMAN 2002), these results are consistent with the regulated neuroendocrine secretion of neuropeptides and other secretory granule contents from the peripheral projections of the CCAP/bursicon neurons within 1 hr AE.

When we examined *CCAP-Gal4/EP(2)2587* flies, which at this temperature (25°) displayed 100% unexpanded wings, we observed two changes in the anti-bursicon immunostaining [Figure 8, B (bottom) and C]. First, the baseline levels of bursicon immunoreactivity were lower than those in the *CCAP-Gal4/+* controls, consistent with the generalized 30–50% decrease in bursicon, CCAP, and mCD8::GFP levels in most lines expressing transgenes in the CCAP/bursicon neurons (see above). Second, there was no depletion of bursicon immunoreactivity during the 1 hr AE in *CCAP-Gal4/EP(2)2587* flies. Thus, the GOF of *miR-310–miR-313* in the CCAP/bursicon neurons inhibited bursicon secretion after eclosion. This inhibition may have resulted from direct

FIGURE 8.—GOF of the *miR-310-miR-313* micro-RNA cluster during an early- to midmetamorphosis critical period prevented bursicon secretion in adults. (A) *In situ* hybridization (magenta) for *miR-310* (antisense LNA probe) in a wandering third-instar larval CNS. Autofluorescence (green) was detected using an FITC/GFP filter set. *EP(2)2587* was expressed under the control of *CCAP-Gal4*. Comparable staining was observed with LNA probes for *miR-312* and *miR-313* (not shown; *miR-311* was not tested). (B) Anti-bursicon staining immediately after eclosion (0 hr AE) and 1 hr after eclosion (1 hr AE) in control flies (*CCAP-Gal4/+*) and flies with *miR-310-miR-313* GOF [*CCAP-Gal4/EP(2)2587*]. (C) Quantification of bursicon levels for the treatments shown in B ($n = 5-6$). * $P < 0.05$; NS, not significant ($P = 0.000224$, one-way ANOVA; Tukey-Kramer multiple-comparison *post hoc* test). The differences in staining intensities were confirmed by blind scoring. (D) Percentage of adult flies with uninflated wings (UEW) or partially

tially expanded wings (PEW) following *miR-310-miR-313* GOF at different developmental stages [$n = 7-119$ (41.0 ± 1.1) per point]. All animals had one copy each of *386Y-Gal4*, *EP(2)2587*, and *tubulinP-Gal80^s*. The shift-up group (black squares, solid line) was collected as embryos at the permissive temperature (19°) and then shifted at the times shown on the x-axis to the restrictive temperature (30°). The shift-down group (open circles, dashed line) was collected as embryos at the restrictive temperature and then shifted at the times shown to the permissive temperature. The curves are fifth-order polynomials—only the center portions of the curves are shown. The gray bar indicates the critical window for the *EP(2)2587* effect on wing expansion: It begins at the shift time that produced wing expansion defects in $\sim 50\%$ of the shift-down animals, and it ends at the shift time that produced displayed wing expansion defects in $\sim 50\%$ of the shift-up animals. Bars: A, $50 \mu\text{m}$; B, $100 \mu\text{m}$.

disruption of the secretory apparatus in the CCAP/bursicon efferents. Alternatively, *miR-310-miR-313* could have altered the electrical activity (or synaptic efficacy) of neurons within the CCAP/bursicon cell population that may provide synaptic input to the subset of neurons with neuroendocrine projections.

Critical window for the *miR-310-miR-313* GOF phenotype: *EP(2)2587* expression did not perturb head eversion, even in homozygous *CCAP-Gal4*, *EP(2)2587* animals. This result was in contrast to the apparent dosage-sensitive disruption of head eversion for a few other lines, including *EP(2)2003*, *EP(2)2237*, and *EP(2)2583* (data not shown). Thus, the effects of *miR-310-miR-313* GOF appeared to be strictly limited to adult-specific functions of the CCAP/bursicon neurons. This could have resulted from an acute block in the expression of one or more proteins required for exocytosis or for *trans*-synaptic activation of the CCAP/bursicon neurons. Alternatively, *miR-310-miR-313* may have altered the earlier development of these cells. To test these hypotheses, we used the TARGET system (McGUIRE *et al.* 2003) to determine the critical period

for the disruption of wing expansion by *miR-310-miR-313* expression in neuropeptidergic cells. We generated a fly line containing *386Y-Gal4* and the *tubulinP-Gal80^s* transgene, which provides ubiquitous expression of a temperature-sensitive Gal80 protein (McGUIRE *et al.* 2003), and crossed these flies to *EP(2)2587*. Gal80^s binds to Gal4 at permissive temperatures ($18^\circ-22^\circ$) and represses Gal4 transcriptional activity. We observed normal wing expansion in $\sim 100\%$ of *tubP-Gal80^s/EP(2)2587*; *386Y-Gal4/+* animals raised throughout development. The inhibition by Gal80^s is released at restrictive temperatures ($27.5^\circ-30^\circ$), and when raised throughout development at 30° , 100% of *tubP-Gal80^s/EP(2)2587*; *386Y-Gal4/+* flies failed to inflate their wings.

To determine the critical period for *miR-310-miR-313* expression, we then conducted temperature-shift experiments. We collected eggs for 24 hr at either 19° or 30° . Groups of animals then were shifted up or down to the other temperature at daily intervals until the adults emerged. After eclosion, we measured the percentage of adults displaying partially or completely uninflated wings (Figure 8D). When the temperature was shifted

down (from 30° to 19°), *miR-310-miR-313* was expressed early and then repressed after the temperature shift. If *miR-310-miR-313* expression was repressed before a critical window opened, then animals would be able to expand their wings. Conversely, when the temperature was shifted up (from 19° to 30°), *miR-310-miR-313* was expressed after the temperature shift. If this occurred after a critical window closed, then animals would be able to expand their wings. Therefore, we defined the opening and closing times for the critical window as the times at which the respective shift-down and shift-up curves each crossed the 50% mark for animals with uninflated wings. These results show that *miR-310-miR-313* expression was effective in blocking wing expansion when the animals were at the restrictive temperature, 30°, between 6.5 and 8 days of age. The curve for timing the onset of the critical window (shift down) displayed a rapid exponential rise that sharply delineated the onset of sensitivity of the CCAP/bursicon cells to *miR-310-miR-313* expression.

The time for the close of the critical window (shift up) was less well defined. In the control experiments by McGUIRE *et al.* (2004), the repression of control transcript expression after shifting to 19° was half-maximal at 15 hr and complete by 36 hr. A similar slow decline likely accounts for the greater variability and shallower curve for the shift-up tests in Figure 8D. When we examined the stage of development of *tubP-Gal80^{ts}/EP(2)2587; 386Y-Gal4/+* animals after 8.7 days at 19°, 71% of the animals were wandering-stage third-instar larvae, and the rest were feeding-stage third-instar larvae ($n = 105$). If the offset of *miR-310-miR-313* occurred ~8–30 hr after the animals were shifted to 19°, then most of these animals would have been in the early stages of pupal development. Therefore, we conclude that the critical window for the effects of *miR-310-miR-313* GOF on the ability of the CCAP/bursicon neurons to secrete bursicon coincides with the first half to middle third of metamorphosis.

DISCUSSION

Analysis of steroid-dependent neuroendocrine cell remodeling through GOF screening: We screened 6097 randomly inserted GOF transposon insertions and identified 30 lines (29 unique loci) that produced wing expansion defects and 5 lines (3 loci) that produced pupal lethality (2 with head eversion or eclosion defects) when crossed to the *CCAP-Gal4* driver. We also identified 19 loci that produced head eversion, eclosion, or wing expansion defects when expressed with the much broader neuropeptidergic cell driver, *386Y-Gal4*, but not *CCAP-Gal4*. These results point to the existence of one or more undefined neuropeptidergic cell types in the *386Y-Gal4* pattern with important roles in the control of head eversion, eclosion, and wing expansion behaviors (supplemental Results and Discussion). Our phenotypic analysis of 17 of the loci that produced the

most severe wing expansion defects with *CCAP-Gal4* suggests functions for these genes in axonal pathfinding, development of competence for developmentally timed neuropeptide secretion, outgrowth of adult-specific neurite projections, neuropeptide expression, and neuronal maintenance. The other lines obtained in the screen revealed several additional genes with putative roles in neuroendocrine cell remodeling.

Combined with LOF data, GOF screens have proved to be an effective method for identification of genes involved in the development of many tissues (RØRTH 1996; RØRTH *et al.* 1998; ABDELILAH-SEYFRIED *et al.* 2000; KRAUT *et al.* 2001; PENA-RANGEL *et al.* 2002; TSENG and HARIHARAN 2002; BRENNECKE *et al.* 2003; MCGOVERN *et al.* 2003; SHERWOOD *et al.* 2004; TELEMAN *et al.* 2005, 2006). However, this method also poses special concerns that are not usually encountered with traditional LOF screens. For example, some genes produce phenotypes in cells in which they are not normally expressed. In many cases, these phenotypes are complementary to LOF phenotypes in other cells, and they are therefore useful for defining gene functions (*e.g.*, ALLAN *et al.* 2003; HEWES *et al.* 2003, 2006). Alternatively, GOF phenotypes may arise from abnormal protein–protein interactions or through the activation of cellular homeostasis machinery, such as the unfolded protein response (RYOO and STELLER 2007). In future studies, it will be important to map the normal expression patterns of these genes and test for complementary LOF phenotypes to identify factors that (1) control remodeling of the CCAP/bursicon during metamorphosis and (2) regulate more general aspects of steroid-dependent neuronal plasticity. Nevertheless, our phenotypic analysis and other published studies provide several insights into their possible functions. Below, we describe a few of the putative associations among these genes.

Micro-RNAs: GOF screens have proved to be effective for identifying functions of micro-RNAs, which are 20- to 22-nucleotide noncoding RNAs that assume diverse gene regulatory roles (KLOOSTERMAN and PLASTERK 2006). Hundreds of micro-RNA genes have been identified through computational prediction and experimental verification (BEREZIKOV *et al.* 2006), yet relatively few micro-RNA functions have been identified. In this study, we isolated 6 insertions, of a total of 58 (10%), that drove expression of three different micro-RNA loci. In other published GOF screens, these 6 insertions produced developmental phenotypes involving regulation of cell growth, differentiation, or apoptosis in the eye imaginal disc, adult dorsal thorax, and external sensory organs and motor axon guidance and synaptogenesis in embryos and larvae (ABDELILAH-SEYFRIED *et al.* 2000; KRAUT *et al.* 2001; PENA-RANGEL *et al.* 2002; TSENG and HARIHARAN 2002; MULLER *et al.* 2005). Other similar screens led to the discovery of micro-RNAs involved in tissue growth and energy homeostasis (BRENNECKE *et al.* 2003; TELEMAN *et al.* 2006). Thus, coupled with target

prediction, LOF experiments, and mapping of micro-RNA expression patterns, GOF screening provides an additional tool for systematic examination of micro-RNA function.

The GOF phenotypes obtained with micro-RNAs in our screen likely result from the repression of unidentified mRNAs that are normally expressed in the CCAP/bursicon neurons. It will be of interest to determine the identity of these target mRNAs and whether the repression by the micro-RNAs occurs normally in other cells or in the CCAP/bursicon neurons at other stages during development. Through *in situ* hybridization of wandering third-instar larval CNS, we detected weak, heterogeneous expression of *miR-279* in the VNC (data not shown) and of *miR-310*, *miR-312*, and *miR-313* in the brain (see RESULTS). Therefore, these micro-RNAs may be involved in the developmental regulation of certain CNS neurons. In a previous study, *miR-311* and *miR-313* were shown to play significant roles in embryonic nervous system development (LEAMAN *et al.* 2005). Interestingly, the *miR-310–miR-313* cluster and the closely related micro-RNAs, *miR-92a* and *miR-92b* were strongly expressed in embryos and then less abundant at later stages (ARAVIN *et al.* 2003; SEMPERE *et al.* 2003). For some of these micro-RNAs, significant downregulation occurred near the embryo–larval and larval–pupal transitions. Our observation of a critical period for *miR-310–miR-313* action during early metamorphosis suggests that the upregulation of some of their target mRNAs was required for the CCAP/bursicon cells to acquire competence to secrete bursicon after eclosion.

Growth factor signaling: Several of the genes identified in our screen are involved in signaling by growth factors. Two of the genes, *foxo* and *Insulin-like receptor* (*InR*), encode components of the insulin signaling pathway. In mammals, multiple forkhead O (FoxO) subfamily transcription factors play important roles in the regulation of neuronal apoptotic death in the context of low-level insulin signaling (GILLEY *et al.* 2003; BARTHELEMY *et al.* 2004; SORIANO *et al.* 2006; BISWAS *et al.* 2007). In *Drosophila*, *foxo* is the only FoxO subfamily member in the genome, and it has been shown to mediate apoptosis in retinal neurons (LUO *et al.* 2007). Here, we found that *foxo* overexpression resulted in evident neurodegenerative phenotypes: reduced and fragmented neurites, reduced cell sizes, and loss of neuronal somata. Interestingly, overexpression of *InR* by *EY(3)00681* in the *386Y-Gal4* pattern also produced pharate lethality. On the basis of these results, we are currently examining the role of insulin signaling in the regulation of neuropeptidergic cell remodeling during metamorphosis (T. GU, T. ZHAO and R. S. HEWES, unpublished observations).

In addition to insulin signaling, the epidermal growth factor receptor (EGFR) signaling pathway is a critical regulator of axon growth and guidance and neuronal

cell survival (DOMINGUEZ *et al.* 1998; DOROQUEZ and REBAY 2006). In this screen, we found two genes with known roles in EGFR signaling during neuronal development, the *pnt* ETS domain transcription factor and the *spen* nuclear corepressor (CHEN and REBAY 2000; KUANG *et al.* 2000). Both *pnt* and *spen* elicited robust head eversion and wing expansion defects when expressed in the *386Y-Gal4* and *CCAP-Gal4* patterns (supplemental Table 3), and *spen* overexpression resulted in the loss of most of the CCAP/bursicon neuron arbor and the disappearance of many abdominal CCAP/bursicon neuron somata (supplemental Table 4). Interestingly, reduced activity of *pnt* can suppress *spen* overexpression-mediated phenotypes (T. ZHAO, T. GU and R. S. HEWES, unpublished observations), which is consistent with a previous report that *spen* functions synergistically with *pnt* in regulation of the EGFR pathway (CHEN and REBAY 2000). These results suggest that EGFR signaling controls aspects of neurite outgrowth and neuropeptidergic cell survival during metamorphosis. Alternatively, *pnt* and *spen* may each influence different signaling pathways that contribute to the development or maintenance of adult CCAP/bursicon projections. In the GOF screen, we also obtained *spitz* (*spi*), which encodes a ligand of the *Drosophila* EGFR. When expressed in the *386Y-Gal4* pattern, *spi* produced pharate adult lethality, presumably due to actions of this ligand on neighboring cells.

Myb proto-oncoprotein-like transcription factors: Of the 14 genes that produced loss of adult-specific neurite projections, two appear to be involved in signaling by Myb-like protein complexes. The *mip120* gene encodes one of five components of the *Drosophila* Myb complex (BEALL *et al.* 2002), and *stonewall* (*stwl*) encodes a Myb-like transcription factor (CLARK and MCKEARIN 1996). In mammals, the apoptotic loss of postmitotic neurons following the loss of trophic support from nerve growth factor (NGF) requires the derepression of multiple transcriptional pathways. These include the FoxO pathway, the c-Jun and c-Jun N-terminal kinase pathway (JNK), and the cell cycle pathway, which involves phosphorylation of retinoblastoma (Rb) proteins as well as the derepression of Myb proteins (BRUNET *et al.* 2001; LIU *et al.* 2004). All three of these pathways converge on Bim, a pro-apoptotic protein that is activated in response to NGF deprivation. Overexpression of either FOXO or Myb family proteins (including STWL) can result in apoptotic cell death (LIU and GREENE 2001; GILLEY *et al.* 2003; BRUN *et al.* 2006). However, the apoptotic response to NGF under physiological conditions may require the combined derepression of all three of these pathways (BISWAS *et al.* 2007). In this screen, *foxo* expression led to loss of CCAP/bursicon cell neurites and somata, while overexpression of *mip120* and *stwl* both led to loss of neurites alone (supplemental Table 4). In the case of *mip120*, the GOF phenotype appears to have resulted from overexpression, rather than misexpression, since

we found that MIP120 was ubiquitously expressed in the larval CNS (supplemental Results and Discussion and T. GU, T. ZHAO and R. S. HEWES, unpublished observations). Likewise, *stwl* was previously shown to be expressed ubiquitously in embryos (TOMANCAK *et al.* 2007). It will be of interest to determine whether these factors function together under physiological conditions to regulate nerve cell growth and survival in this system.

Ecdysteroid signaling: Studies on a variety of cell types in *Drosophila* and *M. sexta* have shown that the metamorphic remodeling of larval neurons is mediated by ecdysteroids. These steroid actions are mediated largely cell autonomously (WILLIAMS and TRUMAN 2005; BROWN *et al.* 2006) through ecdysteroid receptors, which are heterodimers consisting of Ultraspiracle along with one of three isoforms of the ecdysteroid receptor (EcR), EcR-B1, -B2, or -A (KOELLE *et al.* 1991; TALBOT *et al.* 1993; THOMAS *et al.* 1993; YAO *et al.* 1992, 1993). In *Drosophila* neuroendocrine cells as well as in other neuronal cell types, loss of the EcR-B isoforms leads to pruning defects. In contrast, EcR-A is thought to regulate the outgrowth of the adult-specific neurites (TRUMAN *et al.* 1994; TRUMAN 1996; SCHUBIGER *et al.* 1998; LEE *et al.* 2000; BROWN *et al.* 2006; SANTOS *et al.* 2006).

GOF of several of the loci identified in this screen (*cbt*, *CG14438*, *faf*, *foxo*, *klar*, *mip120*, *miR-279*, *spen*, *stwl*, and the genes under the control of *EY(2)04392*, *XP(2)d07339*, *XP(3)d00809*, *XP(3)d02595*, and *XP(3)d04253*) produced defects consistent with the disruption of ecdysteroid-dependent remodeling of the CCAP/bursicon neurons. For *cbt*, we observed pruning on schedule, but formation of the adult central and peripheral adult arbor was retarded, and outgrowth appeared to cease after completion of an initial phase (Figure 6). Interestingly, *cbt* encodes a C2H2 zinc-finger transcription factor (MUNOZ-DESCALZO *et al.* 2005) that functions as a primary ecdysteroid response gene (BECKSTEAD *et al.* 2005). Thus, EcR may directly regulate *cbt* expression, which in turn may regulate the expression of genes involved in controlling neuronal outgrowth.

Ubiquitin–proteasome system: Finally, we identified several genes that have previously been shown to regulate axonal pathfinding or synapse formation in other cell types (*cf.* KRAUT *et al.* 2001). One of these, *faf*, encodes a deubiquitinating protease that contributes to synaptic growth control at the neuromuscular junction (NMJ). The ubiquitin–proteasome system plays a crucial role in axon guidance and synaptogenesis in diverse species (HEGDE 2004). In *Drosophila*, *faf* overexpression at the NMJ produces synapse overgrowth in third-instar larvae (DIANTONIO *et al.* 2001). Similarly, we found that *faf* overexpression produced modest overgrowth, including enlarged varicosities, of larval CCAP/bursicon neurites within the CNS. In contrast, during metamorphosis, *faf* overexpression produced degenerative phenotypes (supplemental Table 4), accompanied by loss of neurite arbor and CCAP/bursicon cell somata.

Thus, the contribution of ubiquitin–proteasome system components to the development of CCAP/bursicon neurons, or in the balance of regulated proteolysis in different subcellular domains, may change as a function of developmental stage.

Taken together, these results provide several molecular clues into how the complex processes of neuronal differentiation, ecdysteroid-dependent regulation of neuronal remodeling, and neuronal growth and maintenance may be coordinated in a single cell type, the CCAP/bursicon cells. The challenge for the future will be to define the biological functions of the factors identified in this screen and their roles in neuronal remodeling.

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