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# Retrograde BMP signaling controls *Drosophila* behavior through regulation of a peptide hormone battery

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## SUMMARY

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 Bursicon β. In *wit* mutants, restoration of *wit* exclusively in CCAP neurons 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.

**KEY WORDS: *Drosophila*, Neuronal development, Neuronal network, BMP signaling, Behavior, Differentiation**

## 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, 2011; 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 the 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, 2011; 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 within 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 (Bursα) and Partner of Burs (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.

## MATERIALS AND METHODS

### Fly stocks

*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$^{GAL4-C155}$* (Lin and Goodman, 1994); *UAS-wit$^{DN}$* [*UAS-wit$^{ΔI}$*; intracellular domain deletion (McCabe et al., 2003)]; *UAS-tkv$^{DN}$* (*UAS-tkv$^{ΔGSK}$*; 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$^{Δ84}$* (Allen et al., 1999)]; *UAS-nEGFP; UAS-CD8-EGFP* (Bloomington Drosophila Stock Center).

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Mutant alleles were kept over *CyO,Act-EGFP* or *TM3,Ser,Act-EGFP*. $w^{1118}$ was the control genotype. Flies were maintained on standard cornmeal food (25°C, 70% humidity).

#### 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.

#### 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.

#### Antibodies

Primary antibodies were: rabbit anti-CCAP [2TB; 1:2000; gift from H. Dircksen (Vomel and Wegener, 2007)]; rabbit anti-Bursα [1:5000; gift from B. White (Luan et al., 2006)]; mouse anti-Bursβ [1:2000; gift from C. Klein (Luo et al., 2005)]; mouse anti-Mip [1A4; 1:1000; gift from A. Mizoguchi (Kim et al., 2010; Yamanaka et al., 2010)]; 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).

#### In situ hybridization probes

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

#### Immunohistochemistry and multiplex fluorescent in situ hybridization

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

#### 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.

#### 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 $\chi^2$ test. Statistical data are presented to the exact *P*-value down to $P<0.0001$. NSD denotes no significant difference at $P>0.05$.

#### 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.

#### 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).

## RESULTS

### Pupal ecdysis requires *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. 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. 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% wild-type, 23.8% partial and 9.5% failed ($n$=21 animals; $P<0.0001$ versus mutant) (Fig. 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. 1D). We confirmed that *UAS-wit* did not rescue in the absence of *Ccap-GAL4* ($P<0.0001$ versus rescued animals) (Fig. 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 ($UAS\text{-}tkv^{Act}$,$UAS\text{-}sax^{Act}$). 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

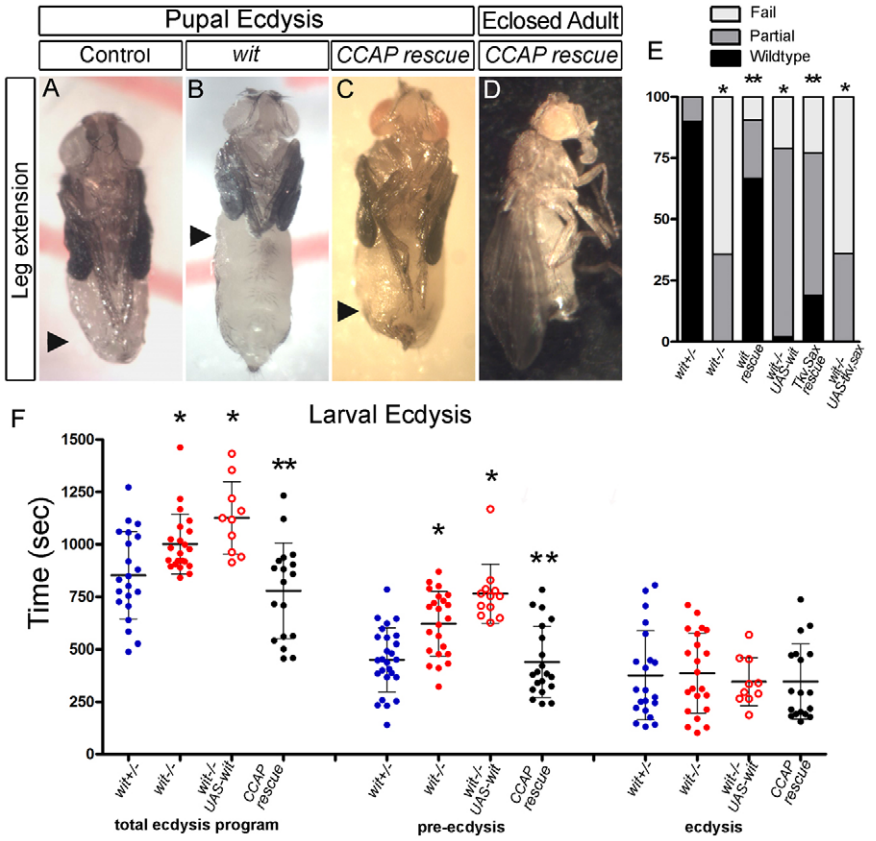


**Fig. 1. *Drosophila* pupal ecdysis requires BMP signaling in CCAP neurons.** (**A-D**) Leg extension (limit indicated by arrowheads) in pharate adults of the indicated genotypes (A-C). *wit* mutants exhibit reduced leg extension (B). *UAS-wit* restoration using *Ccap-GAL4* significantly rescued leg 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$^{B11}$*).

in wild type (see Fig. S1 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. 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.

## 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 the 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. 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). By contrast, ecdysis proper was unaffected, with no significant difference between controls, mutants, rescue controls or rescues (Fig. 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 with 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.

## 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, which is a heterodimer of the Bursα and Bursβ subunits (Ewer, 2005; Honegger et al., 2008) (Fig. 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

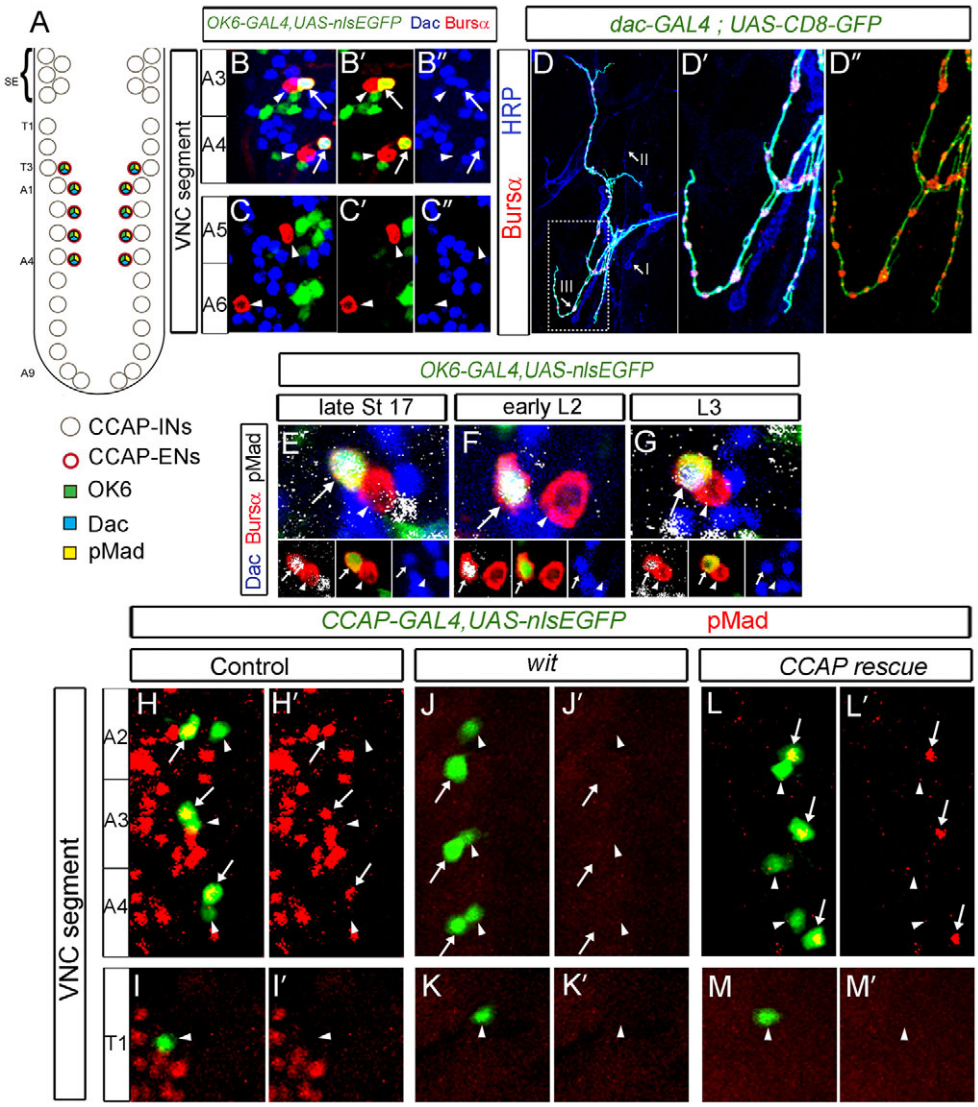


**Fig. 2. CCAP-ENs exhibit active BMP signaling.** (**A**) Distribution of the 36 CCAP-INs and ten CCAP-ENs in subesophageal (SE), thoracic (T) and abdominal (A) segments of the *Drosophila* larval ventral nerve cord (VNC); T3-A4 hemisegments have a CCAP neuron 'doublet' comprising one CCAP-EN and one 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) 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. 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 ten 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. 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. 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. 2E-I). In confirmation of previous reports (Marques et al., 2002), pMad immunoreactivity was eliminated in *wit* mutants (Fig. 2J-K′). As *wit* ecdysis phenotypes were rescued using *Ccap-GAL4* to cell-autonomously restore *wit* (Fig. 1C,D), we examined pMad in those animals. Importantly, we found that, throughout the entire nervous system, pMad was only rescued in the ten CCAP-ENs (Fig. 2L,M). Thus, we conclude that CCAP-ENs relay BMP signaling into the appropriate execution of ecdysis.

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

In *Manduca sexta*, the neuropeptides CCAP and MIP (secreted from the 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 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 ten 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 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. 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. 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. 3E-G). To further support these results, we co-overexpressed dominant-negative BMP receptors ($UAS\text{-}tkv^{DN}$;$UAS\text{-}wit^{DN}$) 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. 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. 3H). Also, Bursβ immunofluorescence intensity declined to 16.6% of controls ($n$=57 CCAP-ENs; $P$<0.0001) (Fig. 3L). Bursα was only subtly reduced to 71.3% of the control intensity in *Mad* mutants ($n$=141; $P$<0.0001).

Interestingly, whereas 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. 3). We examined 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 the activated type I receptors *tkv* and *sax* ($UAS\text{-}tkv^{Act}$,$UAS\text{-}sax^{Act}$) (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

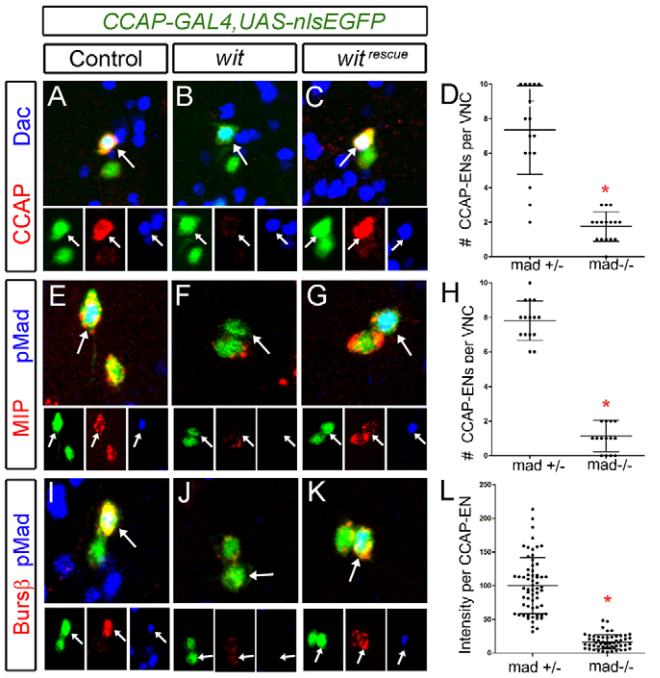


**Fig. 3. Canonical BMP signaling regulates CCAP, Bursβ and Mip in larval CCAP-ENs.** (**A-C**,**E-G**,**I-K**) Images selected from hemisegments A1-A4. Expression of CCAP (A-C), Mip (E-G) and Bursβ (I-K) at L3. CCAP-ENs (arrows) were identified by location and expression of Dac (blue; A-C) or pMad (blue; 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$^{10}$*).

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 with 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) as compared with 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.

## 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., 2010; 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. 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. 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. 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. 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. S1 in the supplementary material) or *wit*-rescued animals (Fig. 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. 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.

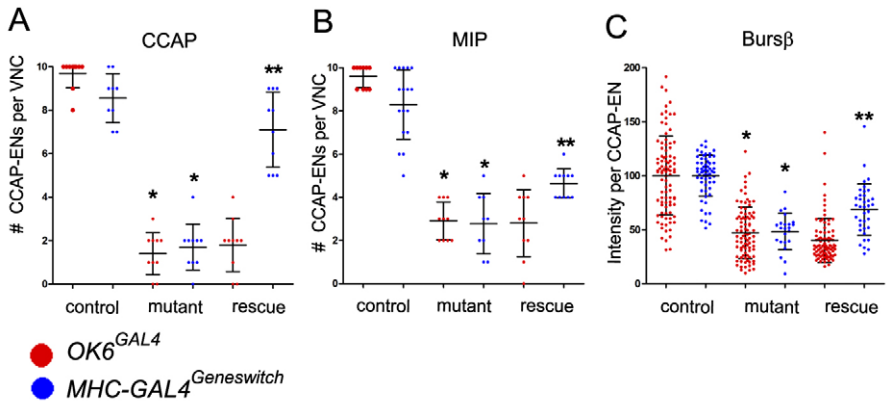


**Fig. 4. Muscle-derived Gbb regulates CCAP, Bursβ and Mip expression.** (**A**,**B**) Scatter plots depict the number of CCAP-ENs that express CCAP (A) and Mip (B) per VNC. CCAP and Mip expression was lost in most CCAP-ENs in *gbb* mutants in genetic backgrounds with *OK6-GAL4* (red dots, neuronal GAL4) 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)].

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

As work in *M. sexta* has 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. 5A,D). In *wit* mutants, leg extension was 4% wild-type, 48% partial and 53% failed ($n$=105; $P$<0.0001 versus control) (Fig. 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. 5D). Next, we tested restoration of pairwise combinations of peptide hormones in *wit* mutants (Fig. 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. 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 S3 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).

## 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.

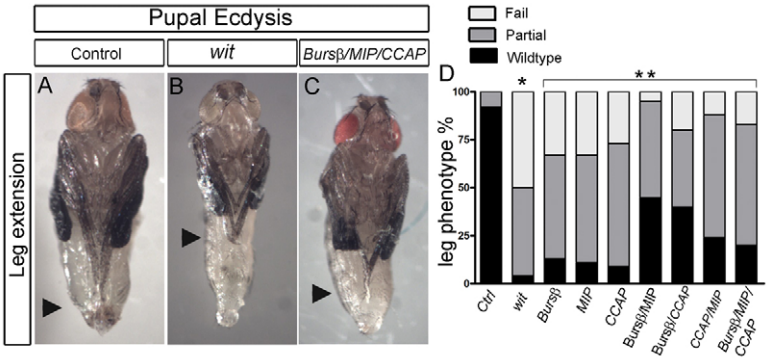


**Fig. 5. Pupal ecdysis is regulated by BMP-dependent peptide hormones in CCAP-ENs.** (**A-C**) *Drosophila* pharate adults showing the posterior limit of leg extension (arrowhead). (A) Controls had wild-type leg extension. (B) *wit* mutants had a deficit in leg extension. (C) Triple restoration of *UAS-burs*β, *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*$^{B11}$).

## 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 hemolymph regulate ecdysis? It has been argued that hemolymph-borne CCAP, Mip and bursicon regulate heart rate, hemolymph pressure and cuticle expansion (Ewer, 2005; Kim et al., 2006b). However, these peptide hormones might 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.

## 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., 2010; 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-Tkv (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 hemolymph. In this regard, it is notable that Ballard et al. (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 hemolymph 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 to be 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 towards the hemolymph and muscle (Atwood et al., 1993; Prokop, 1999; Prokop, 2006). Furthermore, bursicon immunoreactivity is detectable in the hemolymph (Luan et al., 2006). 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, 2011). 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, 2011).

Finally, the differential regulation of Bursα and Bursβ is intriguing because they are believed to only function as a heterodimer (Honegger et al., 2008). Although we do not discount the possibility of functional homodimers, we postulate that the selective BMP dependence of Bursβ might 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 the regulation of only one of its subunits, FSHβ, by the TGFβ family ligand activin (Gregory and Kaiser, 2004; Jorgensen et al., 2004).

## 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, which, 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; Xu and Hall, 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 that 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).

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**Competing interests statement**

The authors declare no competing financial interests.

**Author contributions**

D.W.A. and L.V. conceived all experiments. L.V. conducted the experiments and data analysis. D.W.A. and L.V. wrote the manuscript.

**Supplementary material**

Supplementary material for this article is available at
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**References**


**Aberle, H., Haghighi, A. P., Fetter, R. D., McCabe, B. D., Magalhaes, T. R. and Goodman, C. S.** (2002). wishful thinking encodes a BMP type II receptor that regulates synaptic growth in Drosophila. *Neuron* **33**, 545-558.

**Allan, D. W., St Pierre, S. E., Miguel-Aliaga, I. and Thor, S.** (2003). Specification of neuropeptide cell identity by the integration of retrograde BMP signaling and a combinatorial transcription factor code. *Cell* **113**, 73-86.

**Allen, M. J., Shan, X., Caruccio, P., Froggett, S. J., Moffat, K. G. and Murphey, R. K.** (1999). Targeted expression of truncated glued disrupts giant fiber synapse formation in Drosophila. *J. Neurosci.* **19**, 9374-9384.

**Arber, S., Ladle, D. R., Lin, J. H., Frank, E. and Jessell, T. M.** (2000). ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. *Cell* **101**, 485-498.

**Atwood, H. L., Govind, C. K. and Wu, C. F.** (1993). Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in Drosophila larvae. *J. Neurobiol.* **24**, 1008-1024.

**Ballard, S. L., Jarolimova, J. and Wharton, K. A.** (2009). Gbb/BMP signaling is required to maintain energy homeostasis in Drosophila. *Dev. Biol.* **337**, 375-385.

**Cai, D., Chen, S. and Glanzman, D. L.** (2008). Postsynaptic regulation of long-term facilitation in Aplysia. *Curr. Biol.* **18**, 920-925.

**Clark, A. C., del Campo, M. L. and Ewer, J.** (2004). Neuroendocrine control of larval ecdysis behavior in Drosophila: complex regulation by partially redundant neuropeptides. *J. Neurosci.* **24**, 4283-4292.

**da Silva, S. and Wang, F.** (2011). Retrograde neural circuit specification by target-derived neurotrophins and growth factors. *Curr. Opin. Neurobiol.* **21**, 61-67.

**Di Sanguinetto, S. A., Dasen, J. S. and Arber, S.** (2008). Transcriptional mechanisms controlling motor neuron diversity and connectivity. *Curr. Opin. Neurobiol.* **18**, 36-43.

**Doctor, J. S., Jackson, P. D., Rashka, K. E., Visalli, M. and Hoffmann, F. M.** (1992). Sequence, biochemical characterization, and developmental expression of a new member of the TGF-beta superfamily in Drosophila melanogaster. *Dev. Biol.* **151**, 491-505.

**Eade, K. T. and Allan, D. W.** (2009). Neuronal phenotype in the mature nervous system is maintained by persistent retrograde bone morphogenetic protein signaling. *J. Neurosci.* **29**, 3852-3864.

**Ellis, J. E., Parker, L., Cho, J. and Arora, K.** (2010). Activin signaling functions upstream of Gbb to regulate synaptic growth at the Drosophila neuromuscular junction. *Dev. Biol.* **342**, 121-133.

**Ewer, J.** (2005). Behavioral actions of neuropeptides in invertebrates: insights from Drosophila. *Horm. Behav.* **48**, 418-429.

**Ewer, J., Gammie, S. C. and Truman, J. W.** (1997). Control of insect ecdysis by a positive-feedback endocrine system: roles of eclosion hormone and ecdysis triggering hormone. *J. Exp. Biol.* **200**, 869-881.

**Francis, N. J. and Landis, S. C.** (1999). Cellular and molecular determinants of sympathetic neuron development. *Annu. Rev. Neurosci.* **22**, 541-566.

**Gammie, S. C. and Truman, J. W.** (1997). Neuropeptide hierarchies and the activation of sequential motor behaviors in the hawkmoth, Manduca sexta. *J. Neurosci.* **17**, 4389-4397.

**Gregory, S. J. and Kaiser, U. B.** (2004). Regulation of gonadotropins by inhibin and activin. *Semin. Reprod. Med.* **22**, 253-267.

**Guillemot, F.** (2007). Cell fate specification in the mammalian telencephalon. *Prog. Neurobiol.* **83**, 37-52.

**Haerry, T. E., Khalsa, O., O'Connor, M. B. and Wharton, K. A.** (1998). Synergistic signaling by two BMP ligands through the SAX and TKV receptors controls wing growth and patterning in Drosophila. *Development* **125**, 3977-3987.

**Hall, A. K., Burke, R. M., Anand, M. and Dinsio, K. J.** (2002). Activin and bone morphogenetic proteins are present in perinatal sensory neuron target tissues that induce neuropeptides. *J. Neurobiol.* **52**, 52-60.

**Heanue, T. A., Reshef, R., Davis, R. J., Mardon, G., Oliver, G., Tomarev, S., Lassar, A. B. and Tabin, C. J.** (1999). Synergistic regulation of vertebrate muscle development by Dach2, Eya2, and Six1, homologs of genes required for Drosophila eye formation. *Genes Dev.* **13**, 3231-3243.

**Herrero, P., Magarinos, M., Molina, I., Benito, J., Dorado, B., Turiegano, E., Canal, I. and Torroja, L.** (2007). Squeeze involvement in the specification of Drosophila leucokinergic neurons: different regulatory mechanisms endow the same neuropeptide selection. *Mech. Dev.* **124**, 427-440.

**Hippenmeyer, S., Kramer, I. and Arber, S.** (2004). Control of neuronal phenotype: what targets tell the cell bodies. *Trends Neurosci.* **27**, 482-488.

**Hodge, L. K., Klassen, M. P., Han, B. X., Yiu, G., Hurrell, J., Howell, A., Rousseau, G., Lemaigre, F., Tessier-Lavigne, M. and Wang, F.** (2007). Retrograde BMP signaling regulates trigeminal sensory neuron identities and the formation of precise face maps. *Neuron* **55**, 572-586.

**Honegger, H. W., Dewey, E. M. and Ewer, J.** (2008). Bursicon, the tanning hormone of insects: recent advances following the discovery of its molecular identity. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **194**, 989-1005.

**Jorgensen, J. S., Quirk, C. C. and Nilson, J. H.** (2004). Multiple and overlapping combinatorial codes orchestrate hormonal responsiveness and dictate cell-specific expression of the genes encoding luteinizing hormone. *Endocr. Rev.* **25**, 521-542.

**Khalsa, O., Yoon, J. W., Torres-Schumann, S. and Wharton, K. A.** (1998). TGF-beta/BMP superfamily members, Gbb-60A and Dpp, cooperate to provide pattern information and establish cell identity in the Drosophila wing. *Development* **125**, 2723-2734.

**Kim, N. C. and Marques, G.** (2010). Identification of downstream targets of the bone morphogenetic protein pathway in the Drosophila nervous system. *Dev. Dyn.* **239**, 2413-2425.

**Kim, Y. J., Zitnan, D., Cho, K. H., Schooley, D. A., Mizoguchi, A. and Adams, M. E.** (2006a). Central peptidergic ensembles associated with organization of an innate behavior. *Proc. Natl. Acad. Sci. USA* **103**, 14211-14216.

**Kim, Y. J., Zitnan, D., Galizia, C. G., Cho, K. H. and Adams, M. E.** (2006b). A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. *Curr. Biol.* **16**, 1395-1407.

**Kim, Y. J., Bartalska, K., Audsley, N., Yamanaka, N., Yapici, N., Lee, J. Y., Kim, Y. C., Markovic, M., Isaac, E., Tanaka, Y. et al.** (2010). MIPs are ancestral ligands for the sex peptide receptor. *Proc. Natl. Acad. Sci. USA* **107**, 6520-6525.

**Ladle, D. R., Pecho-Vrieseling, E. and Arber, S.** (2007). Assembly of motor circuits in the spinal cord: driven to function by genetic and experience-dependent mechanisms. *Neuron* **56**, 270-283.

**Lin, D. M. and Goodman, C. S.** (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron* **13**, 507-523.

**Luan, H., Lemon, W. C., Peabody, N. C., Pohl, J. B., Zelensky, P. K., Wang, D., Nitabach, M. N., Holmes, T. C. and White, B. H.** (2006). Functional dissection of a neuronal network required for cuticle tanning and wing expansion in Drosophila. *J. Neurosci.* **26**, 573-584.

**Luo, C. W., Dewey, E. M., Sudo, S., Ewer, J., Hsu, S. Y., Honegger, H. W. and Hsueh, A. J.** (2005). Bursicon, the insect cuticle-hardening hormone, is a heterodimeric cystine knot protein that activates G protein-coupled receptor LGR2. *Proc. Natl. Acad. Sci. USA* **102**, 2820-2825.

**Marques, G.** (2005). Morphogens and synaptogenesis in Drosophila. *J. Neurobiol.* **64**, 417-434.

**Marques, G. and Zhang, B.** (2006). Retrograde signaling that regulates synaptic development and function at the Drosophila neuromuscular junction. *Int. Rev. Neurobiol.* **75**, 267-285.

**Marques, G., Bao, H., Haerry, T. E., Shimell, M. J., Duchek, P., Zhang, B. and O'Connor, M. B.** (2002). The Drosophila BMP type II receptor Wishful Thinking regulates neuromuscular synapse morphology and function. *Neuron* **33**, 529-543.

**Marques, G., Haerry, T. E., Crotty, M. L., Xue, M., Zhang, B. and O'Connor, M. B.** (2003). Retrograde Gbb signaling through the Bmp type 2 receptor Wishful Thinking regulates systemic FMRFa expression in *Drosophila*. *Development* **130**, 5457-5470.

**Martinez-Padron, M. and Ferrus, A.** (1997). Presynaptic recordings from Drosophila: correlation of macroscopic and single-channel K+ currents. *J. Neurosci.* **17**, 3412-3424.

**McCabe, B. D., Marques, G., Haghighi, A. P., Fetter, R. D., Crotty, M. L., Haerry, T. E., Goodman, C. S. and O'Connor, M. B.** (2003). The BMP homolog Gbb provides a retrograde signal that regulates synaptic growth at the Drosophila neuromuscular junction. *Neuron* **39**, 241-254.

**Mesce, K. A. and Fahrbach, S. E.** (2002). Integration of endocrine signals that regulate insect ecdysis. *Front. Neuroendocrinol.* **23**, 179-199.

**Miguel-Aliaga, I., Allan, D. W. and Thor, S.** (2004). Independent roles of the dachshund and eyes absent genes in BMP signaling, axon pathfinding and neuronal specification. *Development* **131**, 5837-5848.

**Miguel-Aliaga, I., Thor, S. and Gould, A. P.** (2008). Postmitotic specification of Drosophila insulinergic neurons from pioneer neurons. *PLoS Biol.* **6**, e58.

**Nishi, R.** (2003). Target-mediated control of neural differentiation. *Prog. Neurobiol.* **69**, 213-227.

**O'Connor-Giles, K. M., Ho, L. L. and Ganetzky, B.** (2008). Nervous wreck interacts with thickveins and the endocytic machinery to attenuate retrograde BMP signaling during synaptic growth. *Neuron* **58**, 507-5018.

**Osterwalder, T., Yoon, K. S., White, B. H. and Keshishian, H.** (2001). A conditional tissue-specific transgene expression system using inducible GAL4. *Proc. Natl. Acad. Sci. USA* **98**, 12596-12601.

**Park, J. H., Schroeder, A. J., Helfrich-Forster, C., Jackson, F. R. and Ewer, J.** (2003). Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and circadian timing of ecdysis behavior. *Development* **130**, 2645-2656.

**Patel, T. D., Jackman, A., Rice, F. L., Kucera, J. and Snider, W. D.** (2000). Development of sensory neurons in the absence of NGF/TrkA signaling in vivo. *Neuron* **25**, 345-357.

**Prokop, A.** (1999). Integrating bits and pieces: synapse structure and formation in Drosophila embryos. *Cell Tissue Res.* **297**, 169-186.

**Prokop, A.** (2006). Organization of the efferent system and structure of neuromuscular junctions in Drosophila. *Int. Rev. Neurobiol.* **75**, 71-90.
**Raftery, L. A. and Sutherland, D. J.** (2003). Gradients and thresholds: BMP response gradients unveiled in Drosophila embryos. *Trends Genet.* **19**, 701-708.
**Ross, S. and Hill, C. S.** (2008). How the Smads regulate transcription. *Int. J. Biochem. Cell Biol.* **40**, 383-408.
**Sekelsky, J. J., Newfeld, S. J., Raftery, L. A., Chartoff, E. H. and Gelbart, W. M.** (1995). Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. *Genetics* **139**, 1347-1358.
**Shi, Y. and Massague, J.** (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.
**Skeath, J. B. and Thor, S.** (2003). Genetic control of Drosophila nerve cord development. *Curr. Opin. Neurobiol.* **13**, 8-15.
**Thummel, C. S.** (2001). Molecular mechanisms of developmental timing in C. elegans and Drosophila. *Dev. Cell* **1**, 453-465.
**Vomel, M. and Wegener, C.** (2007). Neurotransmitter-induced changes in the intracellular calcium concentration suggest a differential central modulation of CCAP neuron subsets in Drosophila. *Dev. Neurobiol.* **67**, 792-808.
**Xu, P. and Hall, A. K.** (2006). The role of activin in neuropeptide induction and pain sensation. *Dev. Biol.* **299**, 303-309.
**Xu, P. and Hall, A. K.** (2007). Activin acts with nerve growth factor to regulate calcitonin gene-related peptide mRNA in sensory neurons. *Neuroscience* **150**, 665-674.
**Yamanaka, N., Hua, Y. J., Roller, L., Spalovska-Valachova, I., Mizoguchi, A., Kataoka, H. and Tanaka, Y.** (2010). Bombyx prothoracicostatic peptides activate the sex peptide receptor to regulate ecdysteroid biosynthesis. *Proc. Natl. Acad. Sci. USA* **107**, 2060-2065.
**Zhao, T., Gu, T., Rice, H. C., McAdams, K. L., Roark, K. M., Lawson, K., Gauthier, S. A., Reagan, K. L. and Hewes, R. S.** (2008). A Drosophila gain-of-function screen for candidate genes involved in steroid-dependent neuroendocrine cell remodeling. *Genetics* **178**, 883-901.
**Zweifel, L. S., Kuruvilla, R. and Ginty, D. D.** (2005). Functions and mechanisms of retrograde neurotrophin signalling. *Nat. Rev. Neurosci.* **6**, 615-625.

**Table S1. Primer sequences for generation of UAS-peptide hormone and in situ hybridization probes**

| Gene | Function* | Sequence (5′ to 3′) |
|---|---|---|
| *Ccap* | Probe | CGCTCCTCCAATTGCTGC |
| | | GGATTTCCCTGAGGCTGC |
| | UAS | AGATCTATGAGAACGTCCATGAGGATT |
| | | TCTAGATCATTTGCTTTCGCGCTCCTC |
| *Mip* | Probe/UAS | AGATCTTATGGCTCACACTAAGACG |
| | | TCTAGAATTAGTTGCTGGGCAACTG |
| *burs*β | Probe | GCATGTCCAGGAACTGCTCT |
| | | TTAATAACGCCCATAGTTGG |
| | UAS | AGATCTATGCATGTCCGGAACTGCTC |
| | | CTCGAGTTAACGTGTGAAATCGCCACA |
| *burs*α | Probe | TTTACGCTCGCCGGGCTTCA |
| | | ACCTGCTCCGCCACGAGAACAA |

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

**Table S2. 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 wit*$^{-/-}$ (*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 (*n*, number of VNCs).

*Compared with pertinent control; NSD, no significant difference.

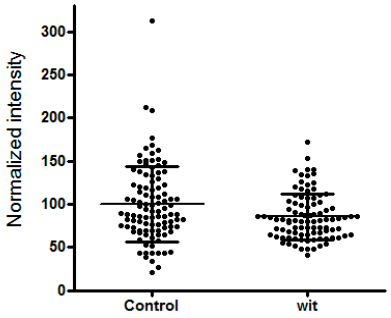

**Table S3. Total number of type III boutons and axonal branch length in controls, *wit* mutants and *wit* rescue**

| Genotype | 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 a shorter projection length compared with 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 with the control. Mean ± s.d.
*Compared with pertinent control; **compared with mutant; NSD, no significant difference.