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

Lyubov Veverytsa and Douglas W. Allan*

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 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.

MATERIALS AND METHODS

Fly stocks
dac-GAL4 (Heanue et al., 1999); OK6-GAL4; wit81; wit811 (Aberle et al., 2002); Ccap-GAL4 (Park et al., 2003); MHC-GAL4; GeneSwitch (Osterwalder et al., 2001); elav-GAL4-C555 (Lin and Goodman, 1994); UAS-witΔI; UAS-witΔIV; intracellular domain deletion (McCabe et al., 2003); UAS-tkvDN (UAS-tkvΔAG3K; GS box and kinase domain deletion); UAS-tkvΔI; UAS-saxΔII; UAS-Δα; UAS-saxΔIII (Haerry et al., 1998); UAS-gbb; gbb1 (Khalsa et al., 1998); MadIR; Df(2L)JS17 [Mad deficiency (Sekelsky et al., 1995)]; UAS-wit2A (Marques et al., 2002); UAS-GluedΔ (UAS-GluedΔI) (Allen et al., 1999); UAS-nEGFP; UAS-CD8-EGFP (Bloomington Drosophila Stock Center).
Mutant alleles were kept over CyO, Act-EGFP or TM3, Ser-Act-EGFP. w^{118} 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. Hsuah, 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-G-C155} and testing immunoreactivity.

**Antibodies**

Primary antibodies were: rabbit anti-CCAP [2T8; 1:2000; gift from H. Dircksen (Vomel and Wegener, 2007)]; rabbit anti-Bursβ [1:5000; gift from B. White (Luan et al., 2006)]; mouse anti-Bursβ [1:200; gift from C. Klein (Luo et al., 2005)]; mouse anti-Mip [1A4; 1:100; 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. Lauehr, 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**

bursβ DIG-uracil-antisense RNA was generated from a 371 bp cloned genomic region (DIG-U-RNA Labeling Kit, Roche). Ccap, Mip and bursα 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 bursα. 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. Images were acquired on an Olympus FV1000 confocal microscope and analyzed afterwards. The other half were timed visually. Both data sets generated identical results.

**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^{112/wit^{B11}}, in which neuronal BMP signaling is eliminated (Marques et al., 2002), we observed a severe deficit 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-Ikv^65, UAS-sax^65). Experiments were performed at 29°C due to the lack of rescue of pMad immunoreactivity in CCAP neuronal nuclei at 25°C, indicating a lack of BMP signaling rescue (see below). At 29°C, pMad immunoreactivity was weakly rescued, although not as strongly as...
in wild type (see Fig. 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 mutants), 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 ecdisis via BMP signaling in CCAP neurons, we suggest two reasons for the partiality of tkv<sup>Act</sup>/sax<sup>Act</sup> rescue. First, constitutive BMP activation in all CCAP neurons caused 80% larval lethality in wit mutants. Also, in controls, Ccap-GAL4-driven tkv<sup>Act</sup>/sax<sup>Act</sup> 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<sup>Act</sup>/sax<sup>Act</sup> 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 ecdisis.

**Larval ecdisis requires wit function in CCAP neurons**

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

At the L2/L3 ecdisis, the entire ecdisis 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-ecdisis and ecdisis proper to the prolonged ecdisis program. Pre-ecdisis 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 is BMP dependent.
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
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 prompted 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 throughout larval and pupal stages, but not in CCAP-INs. (Fig. 2E–G). We examined pMad in those mutants 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).

**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. CCAP 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 (*wit3L/wit3L*), CCAP expression in CCAP-ENs was reduced to 37±26% (*n* = 93 CCAP-ENs) of *wit* heterozygous controls (*wit3L/+; n = 99; *P* = 0.0001). Bursβ expression was reduced to 31±19% (*n* = 67) of controls (*n* = 84; *P* = 0.0001). This finding was newly 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-thy* ΔN; *UAS- tkv* ΔN) 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-thy* ΔN; *UAS- tkv* ΔN; *UAS-sax* ΔN) (Allan et al., 2003) using *Ccapp-GAL4*. This increased CCAP immunofluorescence intensity in CCAP-ENs to 208±19% (*n* = 61 CCAP-ENs) of controls (*n* = 72; *P* = 0.0001), but had no effect on the normally robust CCAP-IN expression of Bursα, which was 103±74% (*n* = 69 CCAP-INs) of
controls (n=62; P=0.79, NSD). Mip and Bursβ expression is mostly absent in CCAP-INS at L3; however, we found that CCAP-INS were capable of increasing peptide hormone expression in response to BMP pathway activation. Quantifying their expression in T3-A8 CCAP-INS (18 CCAP-INS in total), we found that Mip was robustly expressed in 7.9±2.7 T3-A8 CCAP-INS in kn/sux mutant 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-INS rather than CCAP-INS.

**Retrograde Gbb signaling regulates CCAP, Mip and Bursβ in CCAP-INS**

Considerable evidence indicates that peripheral access to the BMP ligand Gbb is required for retrograde BMP signaling in effenter 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-INS, and tested whether muscle, upon which CCAP-INS 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-INS per VNC (n=9 VNCs). In gbb mutants, CCAP was only expressed in 1.7±1.2 CCAP-INS per VNC (n=10; P<0.0001). We restored gbb in muscle using MHC-GAL4;w1118, 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-INS 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-INS per VNC in controls (n=16), falling to 2.8±1.4 CCAP-INS per VNC in mutants (n=9; P<0.0001). This was significantly rescued by muscle-expressed gbb: 4.6±0.7 CCAP-INS per VNC in mutants (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-INS; P<0.0001) relative to controls (n=61 CCAP-INS), and this was significantly rescued by muscle-expressed gbb to 69±24% (n=37 CCAP-INS; P=0.001 versus mutants, P<0.0001 versus controls) (Fig. 4C).

If a peripheral source of Gbb regulates peptide hormone expression in CCAP-INS, 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-GluedDN) 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-INS per VNC in controls (n=10 VNCs) to 1.1±1.6 CCAP-INS per VNC in mutants (n=10 VNCs; P<0.0001 versus control) and downregulated CCAP intensity to 49±13% (n=96 CCAP-INS) 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-restored 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 effenter 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-INS 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-INS.
Finally, we tested triple rescue with nCcap resulted in 11% wild-type, 56% partial and 33% failed leg
wild-type, 50% partial and 5% failed (resulted in 9% wild-type, 64% partial and 27% failed leg
mutant); leg extension with mutants (Fig. 5D). This more dramatically rescued pupal ecdysis restoration of pairwise combinations of peptide hormones in
53% failed (resulted in 13% wild-type, 54% partial and 33% failed
UAS-burs
in mutants and present these in order of efficacy: restoration of wit
behavior. 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.
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 Elosion 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; Prokop, 1999; Prokop, 2006). Furthermore, bursicon vesicles exocytose towards the hemolymph and muscle (Atwood et al., 1993; Prokop, 1999; Prokop, 2006). 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-

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-bursβ, 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) (Manduca 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.
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 bursicron hormone. This would be analogous, and perhaps orthogonal, 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 la 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 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 edysis, rather than acting phasically at edysis to instructively activate edysis behaviors or enable CCAP-ENs to contribute. Such a maintenance role is supported by 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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The authors declare no competing financial interests.

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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.
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References
Drosophila behavior is BMP dependent

DEVELOPMENT


