Retrograde Gbb signaling through the Bmp type 2 receptor Wishful Thinking regulates systemic FMRFa expression in *Drosophila*

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Summary

Amidated neuropeptides of the FMRFamide class regulate numerous physiological processes including synaptic efficacy at the *Drosophila* neuromuscular junction (NMJ). We demonstrate here that mutations in *wishful thinking* (*wit*) a gene encoding a *Drosophila* Bmp type 2 receptor that is required for proper neurotransmitter release at the neuromuscular junction, also eliminates expression of *FMRFa* in that subset of neuroendocrine cells (Tv neurons) which provide the systemic supply of FMRFa peptides. We show that Gbb, a Bmp ligand expressed in the neurohemal organ provides a retrograde signal that helps specify the peptidergic phenotype of the Tv neurons. Finally, we show that supplying *FMRFa* in neurosecretory cells partially rescues the *wit* lethal phenotype without rescuing the primary morphological or electrophysiological defects of *wit* mutants. We propose that Wit and Gbb globally regulate NMJ function by controlling both the growth and transmitter release properties of the synapse as well as the expression of systemic modulators of NMJ synaptic activity.

Key words: FMRFamide, Neurohemal organ, Neuromuscular junction

Introduction

Neuropeptides are a highly diverse group of molecules that modulate specific behaviors and physiological responses in a wide variety of phyla ranging from cnidarians to humans. Although several families are recognized on the basis of conserved sequences, among the most broadly distributed are FMRFamide-related peptides (FaRPs) (Geary et al., 1999; Grimmelikhuijzen et al., 1989; Hinuma et al., 2000; Yang et al., 1985). These peptides share a common C-terminal RFamide sequence and in *Drosophila* are produced from at least five genes, *dromyosuppressin*, *drosulfakinin*, two neuropeptide F-like genes and *FMRFa* (reviewed by Benveniste and Taghert, 1999; Merte and Nichols, 2002). Some of these loci can produce several different peptides exhibiting unique N-terminal sequences by selective proteolytic processing of a proprotein. Sequence analysis of the FMRFa protein suggests that it encodes at least eight unique peptides (Nambu et al., 1988; Schneider and Taghert, 1988; Schneider and Taghert, 1990), three of which have been recovered from tissue extracts (Nambu et al., 1988; Nichols, 1992; Schneider and Taghert, 1988; Schneider and Taghert, 1990). In most insects, FMRF-type peptides mediate their neuromodulatory effects by stimulation of G protein-coupled receptors (Cazzamali and Grimmelikhuijzen, 2002; Chiba et al., 1992; van Tol-Steye et al., 1999; Volterra and Siegelbaum, 1988), but in snails (Cottrell, 1997) and mammals (Lingueglia et al., 1995) there is evidence that they can also directly activate ligand-gated channels.

The physiological processes that FaRPs influence are quite varied. The original invertebrate peptide was isolated from clam ganglia and exhibited marked cardioexcitatory activity (Price and Greenberg, 1977). Other family members, such as Myosuppressins and Sulfakinins, affect spontaneous contractions of the visceral and oviduct muscles and thereby regulate feeding and egg-laying behaviors (Lange and Orchard, 1998; Nachman et al., 1986; Wang et al., 1994; Wang et al., 1995a; Wang et al., 1995b). In *Aplysia*, these peptides can influence learning and memory (Guan et al., 2002; Mackey et al., 1987; Small et al., 1989) while in vertebrates endogenous FaRPs regulate analgesic effects of opiate peptides and influence the electrical activity of some central brain synapses (Askwith et al., 2000; Gayton, 1982; Kavaliers, 1990; Kavaliers and Yang, 1991; Nishimura et al., 2000; Tang et al., 1984; Yang et al., 1985).

In invertebrates, FaRPs can also influence body wall muscle activity. Early studies in the locust suggested that FMRF-like peptides enhance synaptic transmission at the neuromuscular junction (Robb and Evans, 1994), and more recent studies in crustaceans suggest that these peptides modulate presynaptic Ca^{2+}-channel activity (Rathmayer et al., 2002). In *Drosophila*, peptides produced from the *FMRFa* gene can also enhance synaptic efficacy at the neuromuscular junction when perfused

onto standard larval nerve-muscle preparations (Hewes et al., 1998). Such treatment results in a significant increase in muscle contraction or twitch tension. Drosophila *FMRFa* is expressed in many neurosecretory cells, including the Tv neurons that innervate a specialized tissue known as the neurohemal organ (NHO). As the NHO releases products into the hemolymph, FaRPs probably act systemically in a hormone-like fashion to regulate NMJ synaptic activity in vivo (O'Brien et al., 1991; Schneider et al., 1991; Schneider et al., 1993a; Schneider et al., 1993b). Elucidating the mechanisms that control the peptidergic phenotype and activity of particular neurosecretory cells is therefore important for understanding how NMJ activity may be modulated by the neuroendocrine system.

Within the vertebrate nervous system, members of the bone morphogenetic protein (Bmp) subgroup of TGFβ and their cognate receptors have been implicated in controlling several different aspects of neural development and function, including neurulation, morphogenesis, lineage decisions and cellular maturation (reviewed by Mehler et al., 1997). Bmps have also been implicated in neural specification processes. For example, the induction and maintenance of the neuronal cholinergic phenotype in the central nervous system is influenced by Bmp9 (Lopez-Coviella et al., 2000), and trunk neural crest cells are induced to an adrenergic phenotype by Bmp2, Bmp4 and Bmp7 (Reissmann et al., 1996; Varley and Maxwell, 1996). Other Bmps have also been implicated in regulating neurotransmitter expression in sympathetic (Fann and Patterson, 1994; Lo et al., 1998; Schneider et al., 1999), spinal cord (Kalyani et al., 1998), mesencephalic (Jordan et al., 1997; Reiriz et al., 1999), striatal (Hattori et al., 1999) and serotonergic neurons (Galter et al., 1999). In most of these cases, the Bmp is not required for the differentiation of these neurons, rather it helps them obtain their final phenotypic characteristics by inducing the expression of genes specific for the function of that neuron. In *C. elegans*, *daf-7*, a TGFβ-type ligand, is required for maintaining, but not initiating, the expression of chemoreceptors in sensory neurons, thus modulating the chemosensory properties of specific neurons (Nolan et al., 2002).

With these examples as precedent, we sought to determine whether Bmp signals might influence the expression of neuroendocrine phenotypes in *Drosophila*. We and others have recently described a novel *Drosophila* Bmp type 2 receptor, coded for by the *wishful thinking* (*wit*) locus, that is primarily expressed in, and required for, proper nervous system function (Aberle et al., 2002; Marqués et al., 2002). Mutations in *wit* result in pharate lethality caused, in part, by defects in the growth and physiology of motoneuron synapses. We show here that mutations in *wit* also affect the peptidergic phenotype of certain *FMRFa*-expressing cells. In particular, we find that *FMRFa* expression is eliminated in the Tv neurons that contribute to the systemic supply of FMRFa peptides through release at the neurohemal organ. We show that the regulation of *FMRFa* expression in Tv neurons is mediated by the Bmp ligand Gbb, as *gbb* null mutations also eliminate *FMRFa* expression in Tv neurons. Furthermore, we demonstrate that supplying Gbb to the dorsal neurohemal cells restores *FMRFa* expression in Tv neurons. As Tv neuron axons arborize onto the neurohemal cells, this strongly suggests that Gbb signals in a retrograde manner to specify the peptidergic phenotype of Tv neurons. Consistent with this view, we find that overexpression in neuroendocrine cells of Dynamitin or a dominant-negative form of p150/Glued, both components of the Dynactin/Dynein motor complex, also eliminates FMRFa expression in the Tv neurons. Finally, we show that providing *FMRFa* in neuroendocrine cells using the Gal4/UAS system partially rescues the lethal phenotype of *wit* mutants, even though they still exhibit structural and physiological synaptic defects. We suggest that Bmp signaling provides a global cue that not only regulates the growth of the NMJ synapses locally (Aberle et al., 2002; Marqués et al., 2002; McCabe et al., 2003) but also controls their systemic modulation by the neuroendocrine system.

Materials and methods

Drosophila stocks and germline transformations

The wit^{A12}, wit^{B11} and the small deficiency *Df(3L)C175* have been described previously (Marqués et al., 2002). Unless otherwise stated, in all mutant characterizations and rescue experiments *wit* mutants refers to the null heteroallelic combination wit^{A12}/wit^{B11}. The gbb^{1}, gbb^{2} and gbb^{4} alleles are described elsewhere (Wharton et al., 1999) as are the *sax* alleles (Twombly et al., 1996). The *elav*>Gal4 and G14>Gal4, lines were obtained from C. Goodman. The C929 and 386 Gal4 lines were obtained from P. Taghert and 24B>Gal4 was obtained from the Bloomington stock center. The UAS-Dynamitin stocks are described elsewhere (Duncan and Warrior, 2002) as is UAS-ΔGl (Reddy et al., 1997). All germline transformations were carried out by injection into *Df (1) w,* y^{87c27} stock using standard protocols.

Construction of UAS>FMRFa

UAS>FMRFa was generated by synthesis of two 100 bp complementary oligonucleotides that encode the last few amino acids of the *FMRFa* gene. After formation of duplexes, this oligo was inserted into the *Pst*I-*Spe*I sites of an incomplete FMRFa cDNA (Schneider et al., 1991) to generate a full-length coding region. The full-length *FMRFa* gene was excised from the pBluescript clone as an *Eco*RI-*Spe*I fragment and inserted into the *Eco*RI-*Xba*I sites of pUAST (Brand and Perrimon, 1993).

Construction of chimeric receptors

pUC2

pCasper-Ubiquitin (pUC) (Brummel et al., 1994) was digested with *Eco*RI, blunted with Klenow, and religated. The plasmid was cut with *Xba*I and *Stu*I, and a new polylinker was added. Unique sites in the polylinker: 5′ *Not*I, *Sac*II, *Hpa*I, *Avr*II, *Eco*RI, *Xba*I 3′.

pUC-sax (*Stu*I)

A *Stu*I site (AGGCCT) was inserted into the middle of the sax transmembrane domain in pBluescript by site-directed mutagenesis. A *Not*I fragment was inserted into pUC.

Wild-type sequence: GGT CCT TTT CTG GTC ATC
Mutant sequence: GGT CCT TT**A GGC C**TC ATC
Wild-type protein: GPFLVS
Mutant protein: GP **LGL**S
Bold letters indicate changed residues.

pUC-tkv1 (*Stu*I)

A *Stu*I site (AGGCCT) was inserted into the middle of the transmembrane domain of the tkv1 isoform in pBluescript by site-directed mutagenesis. A *Not*I fragment was inserted into pUC.

Wild-type sequence: ATC ATC ATC TCC CTG TCC
Mutant sequence: ATC ATC AT**A GG**C CTG TCC
Wild-type protein: IIISLS
Mutant protein:III**G**LS

pUC2-wit5′ (*Stu*I, *Hpa*I)

*Stu*I (AGGCCT) and *Hpa*I (GTTAAC) sites were inserted into the middle of the wit transmembrane domain in pBluescript by site-directed mutagenesis. A *Hin*dIII (blunted)-*Hpa*I fragment was inserted into the *Hpa*I site of pUC2.

pUC2-wit (*Stu*I, *Hpa*I)

A *Stu*I-*Xba*I fragment of pBluecript-wit5′ (*Stu*I, *Hpa*I) was replaced from pBluecript-wit5′ (*Stu*I, *Hpa*I)

Wild-type sequence: CTT GCC GGT GGA CTC ACA GCC CTC ACA ATC GGC

Mutant sequence: CTT GC**A** GG**C** **CT**A CTC ACA GCC CTC ACA ATC GGC

Wild-type protein: LAGGLTALTIG

Mutant protein: LAG**L**LTALTIG

Wild-type sequence:ATC TTC CTG GCT GTT CAA TAT

Mutant sequence:ATC TTC CTG GCT GTT **A**A**C** TAT

Wild-type protein: IFLAVQY

Mutant protein: IFLAV**N**Y

Chimeric receptors

The chimeric proteins are fused in the middle of the transmembrane domains.

pUC2-sax/wit: a *Not*I-*Stu*I fragment of pUC2-wit was replaced by the extracellular domain fragment of pUC-sax (*Stu*I).

pUC2-tkv1/wit: a *Not*I-*Stu*I fragment of pUC2-wit was replaced by the extracellular domain fragment of pUC-tkv1 (*Stu*I).

pUC2-wit/sax: a *Stu*I-*Xba*I fragment of pUC2-wit5′ was replaced by the sax transmembrane and kinase domain fragment.

pUC2-wit/tkv: a *Stu*I-*Xba*I fragment of pUC2-wit5′ was replaced by the tkv transmembrane and kinase domain fragment.

Antibody staining

The following antibodies were used at the indicated dilutions for characterization of the *wit* and *gbb* mutant phenotypes: monoclonal anti-*lacZ* (Promega) 1/1000; rabbit anti-PSMAD1 (ten Dijke), 1/1000 in embryos and 1/500 in larvae; monoclonal anti-Csp (Zinsmaier) 1/400; and rabbit anti-FMRFamide (Peninsula Laboratories) 1/1000. The Alexa series (Molecular Probes) of secondary antibodies were used for immunofluorescence at 1/500 dilution. Larvae were dissected and fixed in 4% formaldehyde in PBS containing 0.5 mM EGTA, 5 mM $MgCl_2$ for 10-20 minutes at room temperature for pMad and *lacZ* staining. For FMRFamide staining, a protocol was used in which fixation takes place in 0.1 M $NaPO_4$, 0.3% Triton X-100, 0.1%sodium azide, 0.1% BSA for 2-6 hours (McCormick et al., 1999). Staining of all tissues was visualized in a Zeiss Axioplan2 with a CARV unit for confocal microscopy.

Electrophysiology

The standard third instar larval body-wall muscle preparation was used for electrophysiology as previously described (Jan and Jan, 1976; Zhang et al., 1998). Briefly, excitatory junctional potentials (EJPs) in muscles 6 and 7 were elicited by stimulating the innervating motor nerve bundle with a suction electrode in HL-3 solution containing 1 mM $CaCl_2$. The recording microelectrode had an input resistance between 15 MΩ and 25 MΩ. EJPs were acquired and digitized using a PC computer with the use of pCLAMP 8 software (Axon Instruments). The analysis and presentation of figures were conducted on Clampfit (Axon Instruments) and Origin (Origin lab). Samples used for final analysis were obtained from at least five different larvae. ANOVA and Unpaired Student's *t*-test were used for data treatment (mean±s.e.). The resting potential of the muscles was between –65.8±1.2 mV for w control, –64.9±0.6mV for wit^{A12}/*D(3)C175*, and –63.9±1.6 mV for the FRMFa rescue. They were not statistically different from each other (P>0.5).

Results

wit mutants eliminate expression of *FMRFa* in Tv neurons

We previously reported that *wit* mutant flies die during the pharate adult stage and never escape the pupal case. However, some of these individuals exhibit normal peristaltic movements typical of pre-eclosion and, when dissected from the pupal case, are capable of limited spastic movements and will live for several days. During this time we observed that they never inflate their wings, a phenotype that has been ascribed to neuroendocrine defects (Clark et al., 2002; McNabb et al., 1997). As FMRFamide neuropeptides have been shown to affect both synaptic efficacy and muscle twitch tension, and as Bmps have been implicated in vertebrate systems as regulators of final neural phenotypes, we examined whether loss of *wit* signaling disrupted FMRFa expression. Initially, we employed an antibody derived against the peptide FMRFamide. This antibody likely recognizes several classes of FaRPs. As shown in Fig. 1 (compare A with B), we find that in crawling third instar *wit* mutants there is a specific disruption in the accumulation of FaRPs in the Tv neurons and the neurohemal organ. Other FaRP-positive cells such as the subesophageal neurons (SE2) (Benveniste and Taghert, 1999) in the ventral ganglia and several neurons in the brain appear to express FaRPs normally or are only moderately affected. It is important to note that the NHO is composed of two components: the two or three NHO cells and the large varicosities formed by the Tv neurons axons that terminate on the NHO cells. FMRFa accumulation in the Tv neurons appears to be primarily in the large varicosites because, at the RNA level, FMRFa does not appear to be expressed in the NHO cells themselves (O'Brien et al., 1991).

The loss of FaRP staining in the Tv neurons and the NHO varicosities does not necessarily mean that Wit signaling affects transcriptional activation of a FaRP in the these neurons since the effect could potentially occur at several other levels, including production of necessary processing enzymes (Jiang et al., 2000; Kolhekar et al., 1997; Renn et al., 1998). As the *FMRFa* gene is known to be strongly expressed in the Tv neurons that innervate the NHO (O'Brien et al., 1991; Schneider et al., 1993a; Schneider et al., 1993b), we examined the effect of *wit* mutations on the expression of a *FMRFa/lacZ* transgene. The regulatory region of *FMRFa* comprises several different enhancers, including one that produces strong expression in the Tv neurons (Benveniste and Taghert, 1999). As shown in Fig. 1C-F, loss of *wit* signaling specifically eliminates expression of the *FMRFa/lacZ* transgene in the Tv neurons and the NHO. Expression in the subesophageal neurons (Fig. 1D), as well as several other neurons in the brain, remains unaffected (data not shown).

Wit is required in Tv neurons to activate expression of *FMRFa*

As *wit* expression is observed in many neuronal cells, we wished to determine if it is required directly in Tv neurons or whether it might function indirectly via an interneuron signal. To address this issue, we employed three different Gal4 lines that express Gal4 in either all or specific subsets of neuronal cells. The *elav* driver is expressed in most differentiated neurons and, supplying *wit* with this driver restores FaRP

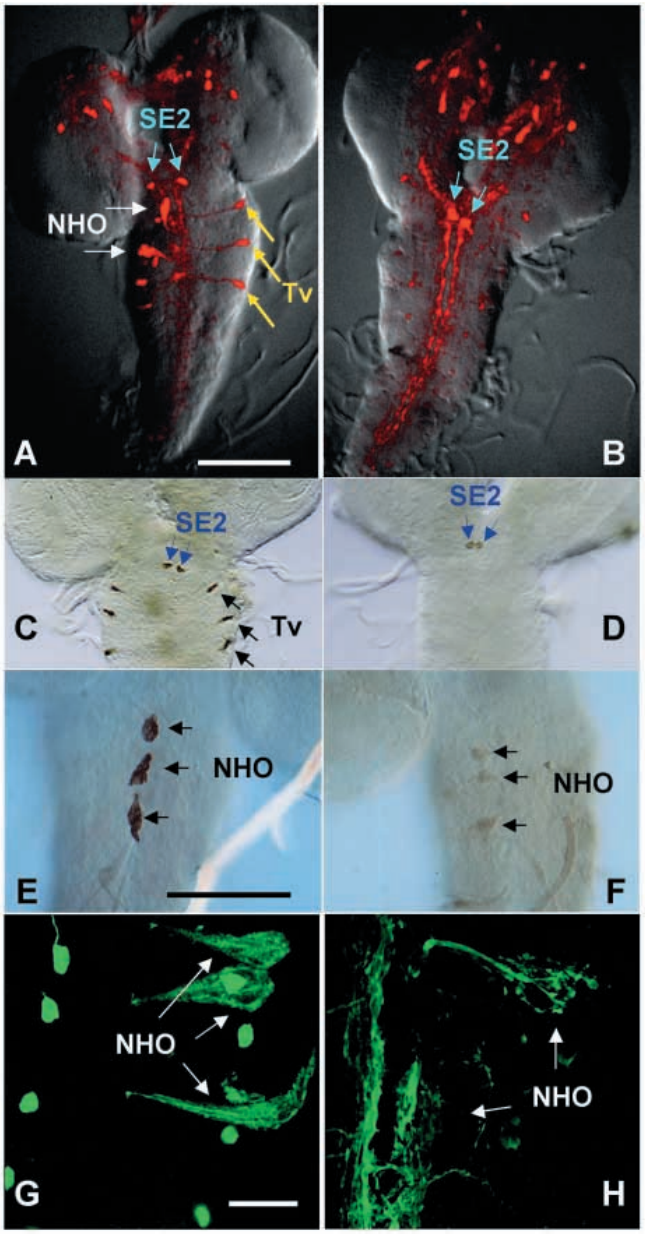


Fig. 1. Mutations in *wit* eliminate systemic expression of *FMRFa*. (A) Wild-type staining of global FMRFamide expression in crawling third instar larval brain and ventral ganglia as revealed by an anti-FMRFamide antibody. A DIC image is overlaid onto the fluorescent image produced by an anti-rabbit Alexa 568-coupled secondary antibody. Two of the three dorsal neurohemal organs (NHO) are identified by white arrows. Three of the six bilaterally symmetric Tv neurons that innervate the NHO are marked with yellow arrows, while the two subesophageal neurons (SE2) are highlighted by light blue arrows. (B) *wit* mutant showing loss of FMRFamide staining in the NHO and Tv neurons. Note that expression in the SE2 neurons is unaffected. In C-F, expression of a *FMRFa/lacZ* transgene (Benveniste and Taghert, 1999) is illustrated in wild-type (C,E) or *wit* mutant animals (D,F). (G,H) τlacZ labeling of the NHO of *wit* mutants (H: *w*; *P{UAS>τlacZ, w*$^{+}$*}/P{ap>Gal4, w*$^{+}$*}*; *wit*^{A12}, *st/wit*^{B11}, *st*) or control animals (G: *w*; *P{UAS>τlacZ, w*$^{+}$*}/P{ap>Gal4, w*$^{+}$*}*; *wit, st/TM6B*) showing that the Tv neurons properly innervate the NHO. Particular cells and structures are marked as above. Scale bars: in A 100 μm for A-D; in E 100 μm for E,F; in G, 20 μm for G,H.

expression in the Tv neurons of *wit* mutants (Fig. 2A). The C929 and 386 lines express Gal4 in much more limited sets of neuroendocrine cells (Taghert et al., 2001), including the Tv neurons but not the NHO (for C929 see Fig. 2C-F). As these drivers also rescue FaRP accumulation in the Tv neurons and the NHO varicosities (2B), we conclude that *wit* is required only in the Tv neurons and is not required to mediate an interneuron signal, nor is it required in NHO cells. In order to confirm that Wit is required in Tv neurons, we took advantage of the variable expression of the OK6 driver in Tv neurons. We have noticed that in addition to the previously described motoneuron expression (Aberle, 2002), this driver is also expressed in a random fashion in some or all of the Tv neurons. We looked at *FMRFa* expression in *wit* mutants rescued with a *witGFP* transgene driven by OK6>Gal4. We find that *FMRFa* expression is variably recovered in different animals, but in all cases (five animals, 17 Tv neurons) the expression of *FMRFa* correlates with the expression of WitGFP in those neurons (Fig. 3). In these experiments, we could also detect WitGFP in the NHO (not shown). In no case did we see WitGFP expression in Tv neurons without *FMRFa* expression.

The absence of systemic FMRFamide could be due to the absence of the Tv neurons or to the lack of proper transcriptional activation of the *FMRFa* gene. Expression of *FMRFa* in Tv neurons is under the control of the transcription factor Apterous (Ap), that directly binds to upstream regulatory elements (Benveniste et al., 1998). We reasoned that Ap activity might be regulated by *Drosophila* Smads directly downstream of Wit (see below). To test this hypothesis we examined *ap* expression in *wit* mutants. Fig. 4 shows the expression of a GFP reporter driven by *ap*>Gal4 in a wild type (Fig. 4A-F) or a *wit* mutant background (Fig. 4G-L). In addition to many other cells, *ap* is expressed in three clusters of four neurons on each side of the ventral ganglion (Fig. 4A,D) (Benveniste et al., 1998). One of these neurons is the *FMRFa*-expressing Tv neuron that innervates the NHO (Fig. 4C,F). In *wit* mutants, FMRFamide expression is absent in the Tv neurons (I), but *ap* expression remains normal (Fig. 4G,J-L). This suggests that *ap* is not a transcriptional target of Wit and also shows that Tv neurons are present in the ventral ganglia of *wit* mutant larvae. This conclusion is further supported by the fact that the NHO proper is still present in *wit* mutants and is roughly the same size as in wild type (Fig. 1E-H). Because much of this organ comprises large varicosities from the Tv axons that arborize onto the NHO cells, it appears that these arbors do form. As with the C929 driver, we could also rescue *FMRFa* expression in *wit* mutant Tv neurons using *ap*>Gal4 and UAS-*wit* (not shown).

Tv neurons require Bmp signals for FaRP expression

We have previously shown that Wit acts to transduce Bmp-type signals (Aberle et al., 2002; Marqués et al., 2002). This requires the Bmp type I receptors Tkv and Sax and results in accumulation of phosphorylated Mad (P-Mad) in motoneurons (McCabe et al., 2003; Rawson et al., 2003). However, recently Wit has been found to be redundant with Punt, a second *Drosophila* Bmp/Activin type II receptor, for transducing activin-like signals through Smad2 in mushroom body neurons (Zheng et al., 2003). In this case, Wit appears to signal in conjunction with Baboon, an activin-like type I receptor

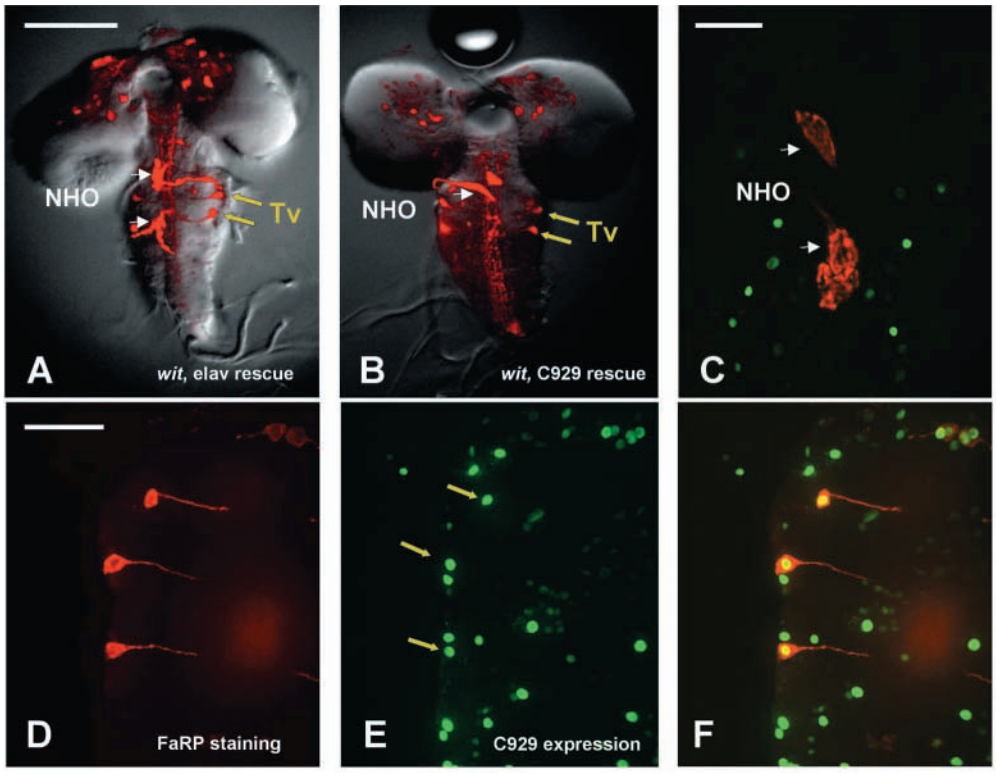


Fig. 2. Expression of Wit in Tv neurons but not the NHO is able to rescue FMRFa expression. Rescue of a *wit* null heteroallelic animal by *elav*>Gal4 (A, *w*; *P{UAS>wit, w⁺}/P{elav>Gal4, w⁺}*; wit^{A12}, *st*/wit^{B11}, *st*) or C929>Gal4 (B, *w*; *P{UAS>wit, w⁺}/P{C929>Gal4, w⁺}*; wit^{A12}, *st*/wit^{B11}, *st*) driving UAS>*wit*. Note rescue of FMRFa expression in the NHO and Tv neurons. (A,B) The third pair of Tv neurons is not in the plane of focus. (C-F) Expression of nuclear *lacZ* using the 929>Gal4 driver is shown in green, while FaRP is in red. (C) Focused on the NHO. Note the lack of lacZ staining in the NHO. (D) Tv neurons showing FMRFa expression (E) The same plane of focus as in D showing nuclear *lacZ*. Yellow arrows identify Tv neuron nuclei. (F) The merged image showing that the C929>Gal4 driver expresses in Tv neurons. Scale bars: in A 100 μm for A,B; panel C 30 μm; panel D (for D-F) 50 μm.

(Brummel et al., 1999). To distinguish whether Wit transduces a Bmp or an Activin type signal to Tv neurons, we initially examined whether P-Mad accumulates in Tv neurons. As shown in Fig. 5A,B), we found clear evidence that these neurons do indeed accumulate P-Mad, suggesting that they receive a Bmp type signal. In a *wit* mutant background, P-Mad accumulation in all ventral ganglion neurons including the Tv neurons is eliminated (Fig. 5C,D).

We also wished to test which type I receptor is required for this process. We found that in the strong heteroallelic $sax^{3}/sax^{Df(2)Hr\text{-}1}$ combination, mutant larvae show greatly reduced expression of FaRPs in the Tv neurons and the NHO (Fig. 5E). As animals containing strong *tkv* alleles do not survive beyond the embryonic stage, we could not test directly for the requirement of Tkv in this process. As expected from the model for signal transduction by TGFβ receptors, a functional receptor complex can be reconstituted by simultaneous expression of two reciprocal chimeras, one containing the extracellular ligand binding domain of the type I receptor and the intracellular kinase domain of the type II receptor and the other formed

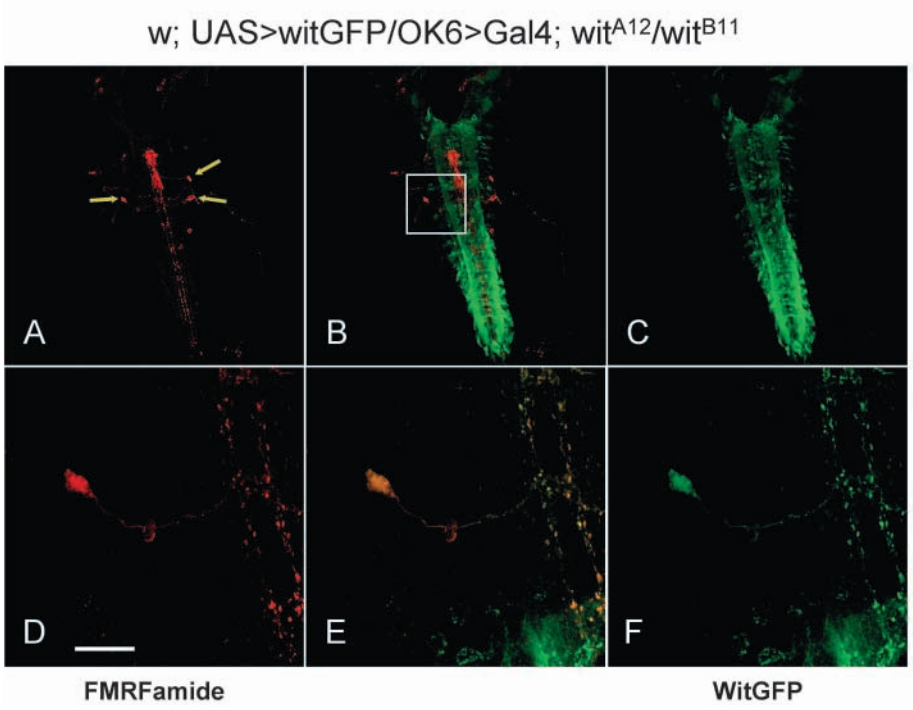


Fig. 3. Cell-autonomous rescue of FMRFamide expression by Wit. Larvae of the genotype *w*; *P{UAS>witGFP, w⁺}/P{OK6>Gal4, w⁺}*; wit^{A12}, *st*/wit^{B11}, *st* were stained for FMRFamide and Wit (GFP). *z*-stacks are shown. (A-C) Dorsal view with anterior on top, showing the chimeric rescue of FMRFamide expression in Tv neurons. The three (out of six possible) FMRFamide-positive Tv neurons are marked by yellow arrows (A). Note the predominantly motoneuron GFP staining that reports driver expression. (D-F) High magnification view of the frame in B. Note co-expression (yellow in E) of WitGFP (F) and FMRFamide (D). (B,E) Merged images. Scale bar: 100 μm in A-C; 20 μm in D-F. Dorsal view, anterior upwards.

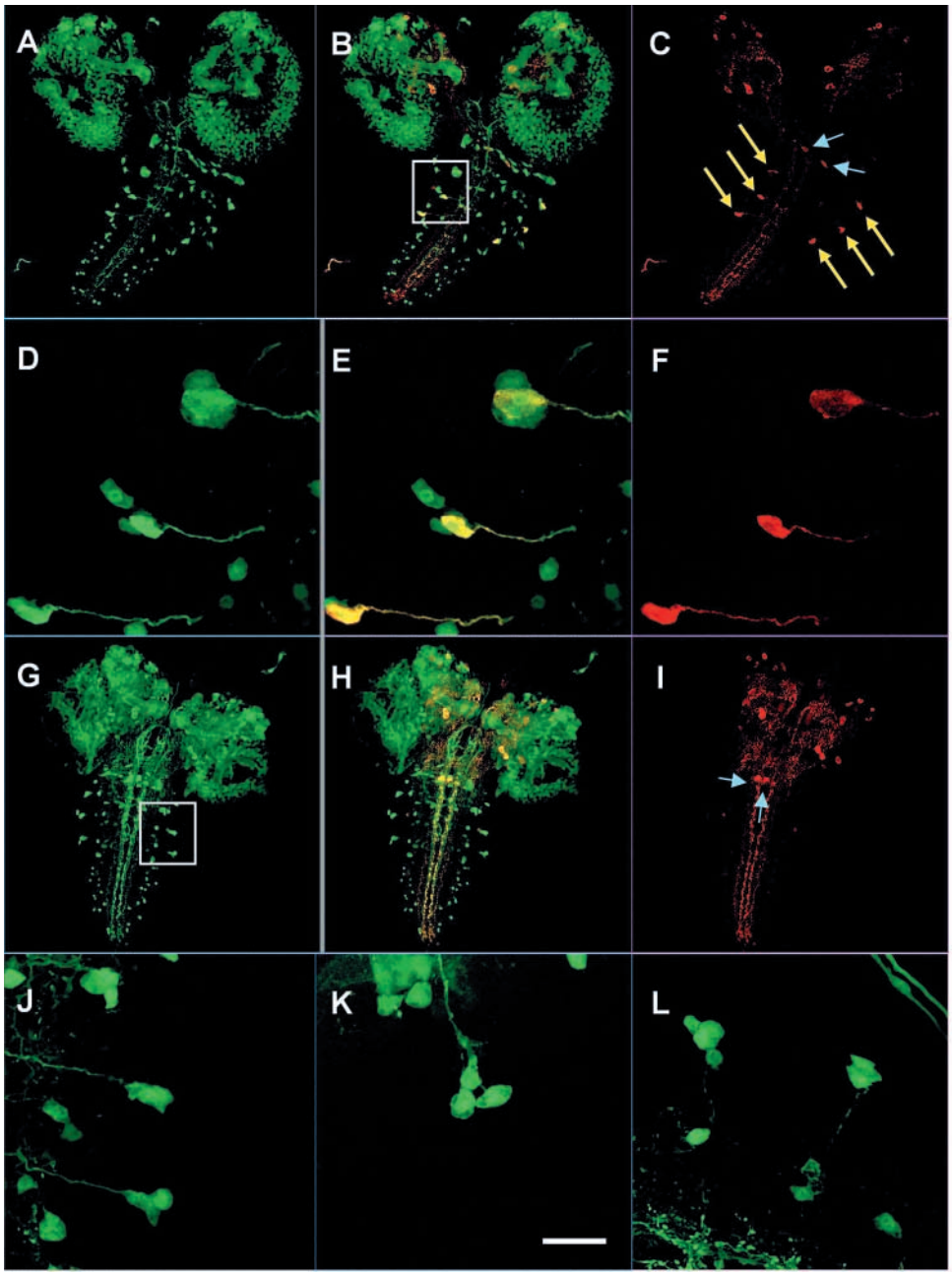


Fig. 4. Mutations in *wit* do not affect *apterous* expression or Tv neuron viability. Third instar larval brains are shown with GFP in green and anti-FMRFamide in red. FMRFamide staining in Tv and subesophageal neurons is indicated by yellow and blue arrows, respectively. (A-F) *w; P{aP>Gal4, w$^+$}/{UAS>GFP, w$^+$}; wit/TM6B*. (D-F) Higher magnification of the area framed in B. (G-L) *w; P{aP>Gal4, w$^+$}/{UAS>GFP, w$^+$}; wit^{A12}/wit^{B11}* animals. (J) High-magnification view of the area framed in G. (K,L) High-magnification views of Ap-positive clusters in two other mutant animals. Scale bar: 100 μm for A-C,G-I; 20 μm for D-F,J-L.

by the extracellular domain of the type II receptor and the intracellular domain of the type I receptor (Feng et al., 1995). This functional complementation is a strong indication that the type I and type II receptors that make up the chimeras act as partners in vivo. We found that the requirement for Wit can be circumvented when a chimeric pair of receptors, one containing the extracellular domain of Tkv fused to the intracellular domain of Wit, and the other composed of the extracellular domain of Wit fused to the intracellular domain of Tkv (see schematic in Fig. 5), are co-expressed in a *wit* mutant background. By themselves, these receptors are unable to rescue either NMJ defects or *FMRFa* expression (data not shown). However when both are expressed using ubiquitin promoters, then we observe rescue of FaRP expression (Fig. 5F), strongly supporting that Tkv acts in vivo with Wit to activate *FMRFa* expression in Tv neurons. An equivalent set of chimeras of Sax and Wit rescues neither defect (data not shown). This observation is consistent with our previous finding that ubiquitous expression of Tkv alone can rescue *sax* mutants, while ubiquitous Sax cannot rescue *tkv* mutants (Brummel et al., 1994). Thus, we conclude that, although Sax is required for both NMJ growth and *FMRFa* expression, it is not able by itself to transduce sufficient signal for these two processes. Instead these data strongly support the notion that Wit is supplying a Bmp signal through both Tkv and Sax.

Although these findings demonstrate that reception of a Bmp signal is an essential component of *FMRFa* gene regulation in Tv neurons, they do not rule out that an activin-type signal may also be received and required by the Tv neurons to stimulate *FMRFa* expression. To probe this issue, we examined *FMRFa*

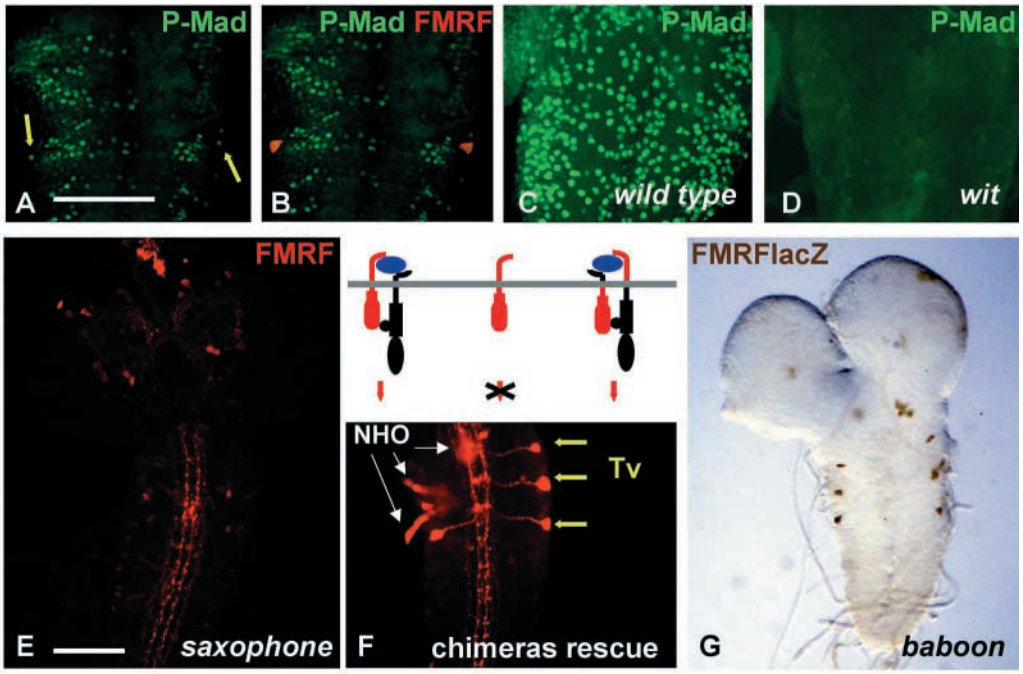


Fig. 5. Bmp signals are received by Tv neurons. (A) A single plane of focus showing P-Mad accumulation in subsets of neurons in the thoracic region of the ventral ganglion. Two Tv neurons are highlighted with yellow arrows. (B) The same image as in A except FMRFa expression is overlaid to highlight the two Tv neuron cell bodies. (C) A compressed stack of optical images through the thoracic region of the ventral ganglion showing all P-Mad accumulating cells. Most of these are motoneurons as revealed by double staining with other markers (data not shown). (D) An equivalent stack to that in C but through a wit^{B11}/wit^{A12} mutant animal. Note the absence of P-Mad accumulation in all cells of the ventral ganglion. (E) FMRFa expression in a $sax^{3}/sax^{Df\,Hr-1}$ mutant animal. (F) Rescue of FMRFa expression in the Tv neurons (yellow arrows) and the NHO (white arrows) of wit^{A12}/wit^{B11} animals ubiquitously expressing the Wit^{EC}/Tkv^{IC} and Tkv^{EC}/Wit^{IC} pair of chimeric receptors. The schematic above F depicts, from left to right, the situation in wild-type animals in which a heterodimer of Wit (black) and Tkv (red) binds Gbb (blue) resulting in activation of the pathway (red arrow); *wit* mutants in which there is no ligand binding nor pathway activation; and *wit* mutants supplemented with the reciprocal chimeras. In this case, the intracellular domains of Wit and Tkv are brought together by Gbb binding to the extracellular domains of the receptors, resulting in pathway activation analogous to the wild-type situation. See Feng et al. (Feng et al., 1995) for more details. (G) *FMRFa/lacZ* expression in the brain and ventral ganglia of a $babo^{P1164}$ homozygous larva. Scale bars: in A, 50 μm for A-D; in E, 50 μm for E-G.

expression in *baboon* mutant third instar larvae. As shown in Fig. 5G, we found that expression of the *FMRFa/lacZ* transgene is still evident in *babo* mutant larvae, demonstrating that an activin-type signal is not required for *FMRFa* expression.

Retrograde Gbb signaling is required for FaRP expression in Tv neurons

The *Drosophila* genome contains seven TGFβ type ligands. Three of these, Dpp, Screw and Gbb, have been shown to transduce Bmp-type signals (Mad) and to use the type I receptors Tkv and Sax (Brummel et al., 1994; Haerry et al., 1998; Nguyen et al., 1998). Two others, Activin and Activin-like protein, transduce signals through Smad2 (T.E.H. and M.B.O., unpublished). The signaling pathways used by Maverick and Myoglianin remain untested. Among the three Bmp-type ligands, Gbb seemed a likely candidate for controlling expression of *FMRFa* as it is broadly expressed, at least in embryos (Doctor et al., 1992; Wharton et al., 1991), and can signal through Wit to regulate P-Mad accumulation in motoneurons and tissue culture cells (McCabe et al., 2003). As shown in Fig. 6A, *gbb* is strongly expressed in the larval brain lobes and much more weakly in the ventral ganglia. Interestingly, we also note that *gbb* shows enriched expression in the NHO relative to other ventral ganglia neurons (Fig. 6A). Thus, Gbb is expressed in the correct place to be a *FMRFa* regulating ligand.

To test directly whether *gbb* is required for *FMRFa* expression, we examined *gbb* mutant larvae for FaRP expression. As shown in Fig. 6C,D, we found that, although the gbb^{1}/gbb^{4} hypomorphic combination still expresses FaRPs, the stronger gbb^{1}/gbb^{2} mutant larvae show a selective loss of FaRP expression in Tv neurons and the NHO, strikingly similar to that exhibited by *wit* mutants. When Gbb is re-supplied to all neurons using the pan neuronal *elav*>Gal4 driver (Fig. 6E), expression of FaRP is restored; if the muscle specific G14>Gal4 driver is utilized, only weak expression is occasionally seen in some Tv neurons (Fig. 6F).

The fact that *gbb* is expressed in the NHO onto which the Tv neuron axons terminate suggested that perhaps Gbb signals to these neurons via a retrograde mechanism. To address this issue, we took advantage of the observation that the 24B>Gal4 driver, while expressed in muscles, is also specifically expressed in the NHO cells (Fig. 7D-F) but not in the Tv neurons themselves (Fig. 7A-C). When 24B>Gal4 is used to express Gbb in the NHO, we found that FaRP expression is restored in the Tv neurons (Fig. 7G), while as shown above, use of the muscle specific driver G14>Gal4 does not rescue (Fig. 6F).

Although these results strongly suggest that Gbb can signal in a retrograde fashion from the NHO to control expression of *FMRFa* in the Tv neurons, they do not directly examine whether this is the case in vivo. To address this issue, we overexpressed a truncated form of p150/Glued (ΔGl) in neuroendocrine cells. Overexpression of this protein interferes with assembly of the Dynein retrograde motor with its cargo leading to the development of 'roadblocks' in which transported proteins collect at jams within axons (Martin et al., 1999). These jams can ultimately interfere with both anterograde and retrograde transport. When we overexpress

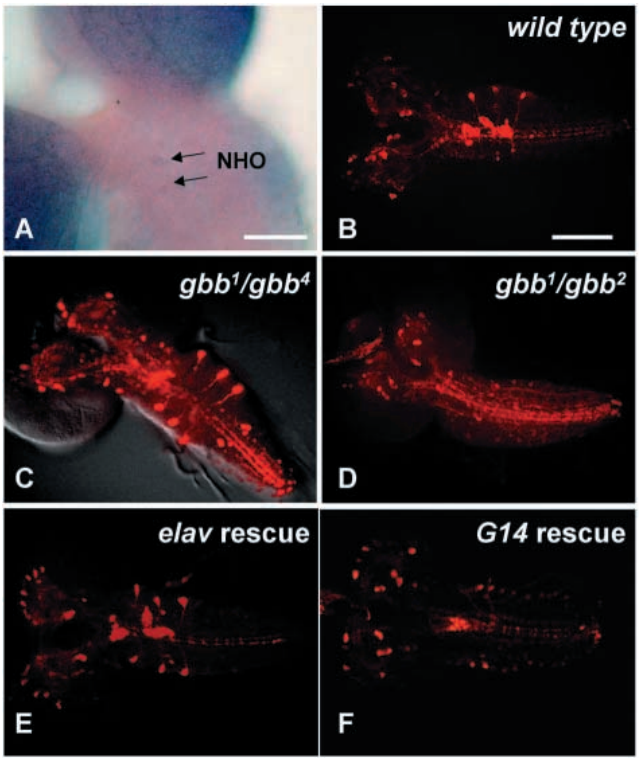
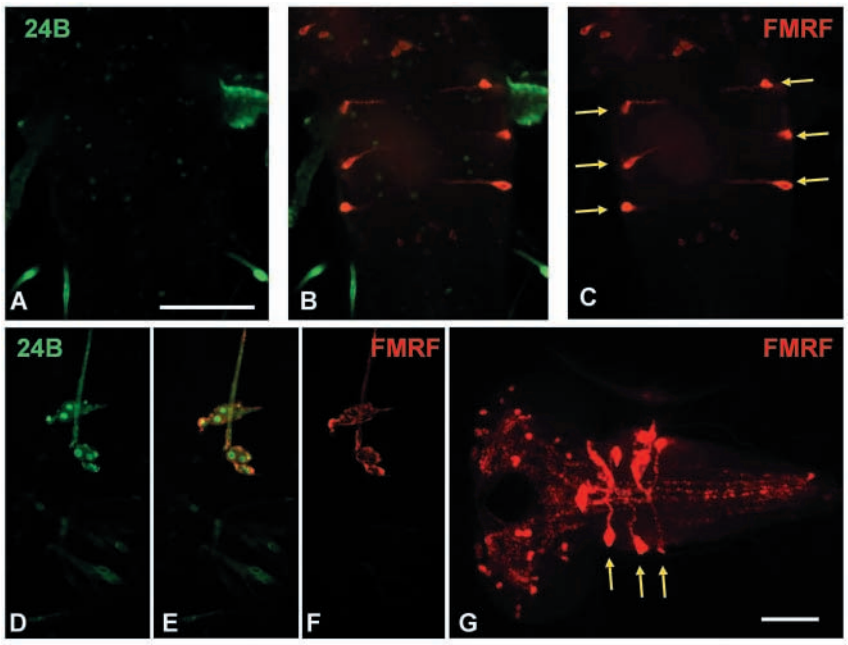


Fig. 6. Gbb is required for FMRF expression. (A) in situ hybridization of third instar brain and ventral ganglia with a *gbb* probe. The arrows indicate the NHO. (B-E) FMRF expression in (B) wild-type, (C) *gbb*^{1}/*gbb*^{4} hypomorph and (D) *gbb*^{1}/*gbb*^{2} null mutant animals. (E) Rescue of FaRP expression in a *gbb*-null background by expression of Gbb using the neuron specific *elav*>Gal4 driver (*w*; *P{UAS>gbb, w*$^{+}$*}, gbb*^{1}/*gbb*^{2}*; P{elav>Gal4, w*$^{+}$*}*/+), (F) Lack of FaRP rescue in a *gbb* null background using the muscle specific G14>Gal4 driver to express Gbb (*w*; *P{UAS>gbb, w*$^{+}$*}, gbb*^{1}/*gbb*^{2}, *P{G14>Gal4, w*$^{+}$*}*). Scale bars: in A, 50 μm for A; in B, 100 μm for B-F Anterior towards the left, dorsal views.



ΔGl in neuroendocrine neurons using the C929>Gal4 driver, these animals survive to the pupal stage and about 50% eclose. However, many eclosed adults exhibit uncoordinated movements and some do not inflate their wings (Fig. 9E) reminiscent of *wit* mutants (not shown). When we examine these larvae for FaRP expression, we find that it is dramatically reduced in the Tv neurons as well as the NHO relative to wild type (Fig. 8A). When the 24B>Gal4 line is used to express ΔGl in the NHO, but not in Tv neurons, then no effect on FaRP expression is seen (Fig. 8B). In these experiments, other FaRP-positive cells in the brain show equivalent staining in both wild type and the ΔGl-expressing line.

These results indicate that the Tv neurons are particularly sensitive to a block in Dynein function consistent with a requirement for retrograde transport in specifying their peptidergic phenotype. To ensure that this is not an artifact of blocking another function of P150/Glued, we also overexpressed p50/Dynamitin. In mammals, overexpression of the Dynamitin subunit of the Dynactin complex has been shown to dissociate Gl from the Dynactin complex, thereby disrupting cargo attachment to the Dynein motor (Echeverri et al., 1996; Eckley et al., 1999). In *Drosophila*, phenotypes consistent with Dynein motor blocks have also been seen when Dynamitin is overexpressed (Duncan and Warrior, 2002; Januschke et al., 2002). When we use C929>Gal4 to overexpress *Drosophila* Dynamitin in Tv neurons, we see a similar loss of FaFR expression as was found for ΔGl (Fig. 8C).

To demonstrate that these effects are not the result of interference with P-Mad transport from the cell cytoplasm to the nucleus, we also expressed ΔGl in the embryonic gut using the Y45>Gal4 driver (Wharton et al., 1999). We have previously shown that P-Mad accumulates extensively in the midgut beginning at stage 15 of embryogenesis (Marqués et al., 2002). Overexpression of ΔGl did not interfere with P-Mad accumulation in the midgut (Fig. 8D versus 8E), nor did it interfere with P-Mad accumulation in dorsal cells when a ubiquitous Gal4 driver such as *daughterless* is used (Fig. 8F versus 8G). The only tissue showing a loss of P-Mad staining when using the ubiquitous *da*>Gal4 line is the CNS (McCabe et al., 2003). We conclude that P-Mad accumulation in neurons is most sensitive to disruption of Dynein motor function, consistent with the notion that Bmp signaling in these cells requires retrograde transport of at least one component as part of its signal transduction mechanism.

Fig. 7. Expression of Gbb in the NHO rescues FMRFa expression in Tv neurons. Nuclear *lacZ* accumulation from a UAS *nuclacZ* driven by 24B>Gal4 is shown in green, while FaRP expression is in red. The 24B driver is not expressed in the Tv neurons (A-C) but it is expressed in the NHO cells (D-F). (G) Rescue of FaRP expression in Tv neurons (yellow arrows) using the 24B>Gal4 driver to drive UAS>*gbb* (*w*; *P{UAS>gbb, w*$^{+}$*}, gbb*^{1}/*gbb*^{2}*; P{24B>Gal4, w*$^{+}$*}*/+). Dorsal view in all panels. (A-F) Anterior upwards; (G) anterior towards the left. Scale bars: in A, 50 μm for A-F; in G, 50 μm for G.

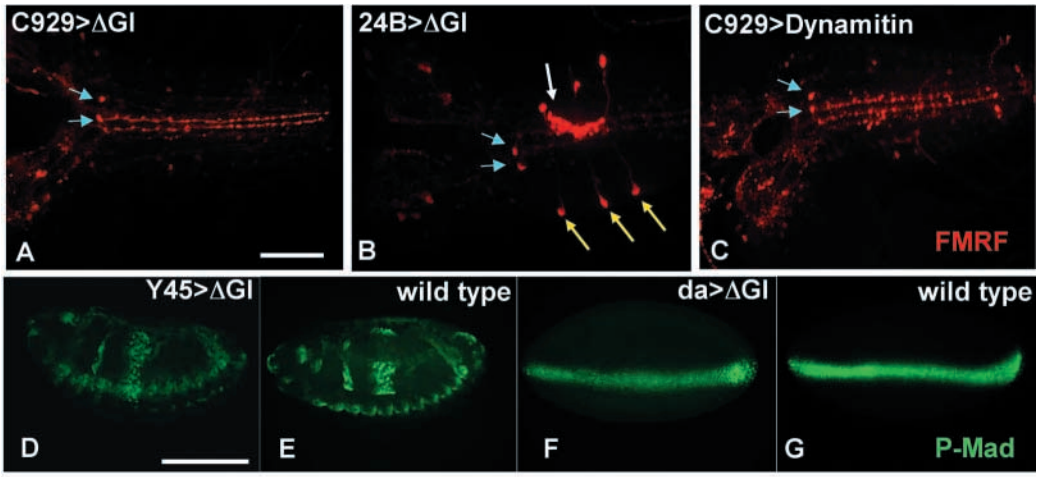


Fig. 8. Disruption of the Dynein motor complex selectively interferes with FMRFa expression in Tv neurons. (A) FaRP expression in third instar larval brain and ventral ganglia in which ΔGl is produced in neuroendocrine cells using the C929 driver. Note lack of staining in the NHO and Tv neurons, SE2 unaffected (light-blue arrows). (B) Expression of ΔGl in the NHO using the 24B driver does not interfere with FaRP staining in Tv neurons (yellow arrows) or NHO (white arrow). (C) FaRP staining in animals expressing p50/Dynamitin using the C929 driver. P-Mad accumulation in the midgut of Y45>Gal4; UAS-ΔGl (D) and wild-type embryos (E). P-Mad accumulation in future amnioserosa cells of *da*>Gal4; UAS-ΔGl (F) and wild-type embryos (G). Scale bars: in A, 50 μm for A-C; in D, 200 μm for D-G. Anterior leftwards in all panels. (A-C,F,G) Dorsal views; (D,E) lateral views.

Overexpression of FMRFa partially rescues *wit* mutants

As FaRPs are known to enhance synaptic transmission at the neuromuscular junction, we wished to determine if re-supplying *FMRFa* alone could partially ameliorate the *wit* mutant phenotype. We have previously shown that *wit* mutants exhibit structural defects at active zones and are defective in synaptic transmission (Aberle et al., 2002; Marqués et al., 2002). Our rationale was that, although re-supply of FaRPs would probably not rescue the primary structural defect of the synapses, it might enhance synaptic transmission enough to allow some animals to eclose. For this purpose we constructed a UAS>*FMRFa* transgene and expressed it in neuroendocrine cells using C929>Gal4. At 25°C we find that ~80% (n=65) of *wit* mutant pharate adults can partially escape from the pupal case (Fig. 9B). In the absence of *FMRFa* expression less than 0.5% of *wit* mutants (0 of 212) open the operculum. At 28°C about 30% of the females can fully eclose (Fig. 9C) and some inflate their wings (Fig. 9D). While these animals move fairly well they usually die within several days without producing progeny. Although many of these animals are able to eclose, we see no rescue in the size of the synapse (Fig. 9F,G) nor is the primary defect in synaptic transmission rescued (Fig. 9H,I). We conclude therefore, that the inability of *wit* mutant animals to eclose is the result of at least two defects, one in NMJ synaptic growth and neurotransmitter release, and the other in the production of systemic FaRPs that act as neuromodulators at the NMJ.

Discussion

Wit and Gbb are global regulators of synaptic function

Maintaining constant synaptic efficacy at the neuromuscular junction throughout development requires a precise coordination of muscle and synapse growth. In addition, NMJ function is likely to be modulated by both behavioral and physiological inputs that derive from stage specific developmental signals and changes in the environment. We have previously shown that Bmp signaling through the type II receptor Wit is a key regulator of NMJ synaptic growth (Aberle et al., 2002; Marqués et al., 2002). More recently, we have found that the Bmp-like ligand Gbb signals through Wit to mediate synapse growth (McCabe et al., 2003). In this report, we show that the Wit pathway specifically turns on the systemic component of FMRFamide in Tv neurons. Absence of either the ligand Gbb or the type II receptor Wit results in loss of expression of the FMRFa precursor protein in Tv neurons.

In *Drosophila* FMRFamide peptides have been shown to enhance synaptic transmission and muscle twitch tension when perfused onto standard larval nerve-muscle preparations (Hewes et al., 1998); however, their in vivo role(s) are not known as no mutations in the *FMRFa* gene have been identified. As with most neuropeptides, FaRPs are thought to act as neuromodulators and neurohormones. The Tv-produced FaRPs are released into the hemolymph through the neurohemal organ and hence are able to act on every tissue in the animal that is not blocked to hemalymph contact. We have previously hypothesized that the lethality of *wit* mutants is due to the lack of proper synaptic transmission at the NMJ, resulting in the animals not being able to eclose from the pupal case (Marqués et al., 2002). The lack of systemic FMRFamide described here would be expected to further decrease synaptic efficiency and the ability of *wit* mutants to eclose. The fact that loss of FMRFa does contribute to the lethal phenotype is supported by the partial rescue of *wit* mutants by overexpression of *FMRFa*. These results are consistent with the view that in vivo, FMRFa peptides probably enhance NMJ synaptic activity similar to their in vitro documented effects on standard larval electrophysiological preparations.

It is important to note that although the lethal phenotype is partially reversed, the morphological and physiological synaptic defects reported for *wit* mutants are not rescued by overexpression of *FMRFa*. The simplest interpretation is that the excess of FaRPs enhances the efficiency of *wit* mutant synapses in vivo without correcting the underlying developmental defects. Although one might expect a

significant improvement of the electrophysiological phenotype, this is not detected (Fig. 9), probably because the excess FaRPs are either washed off the preparation during standard dissection prior to recording or act for only short periods.

Although we have argued that the FMRF rescue effect is the result of enhance synaptic transmission at the NMJ, we can not exclude the possibility that the rescue of eclosion by *FMRFa* expression in *wit* mutants is due to a central effect on ecdysis regulation, and not to a peripheral effect on the NMJ. The driver used, C929, expresses *FMRFa* in all neurosecretory cells, so extra FMRFamide is conceivably produced in the CNS. However, it is important to recognize that *wit* mutants are defective only in the systemic, Tv-secreted form of FMRFamide. Although FMRFamide has a well-studied effect in modulating visceral and somatic muscle contraction (Nichols, 2003), no effect for FMRFamide in triggering eclosion has been uncovered (Fuse and Truman, 2002; Zitnan et al., 2003). In fact, FaRPs accumulate in a model of developmental arrest in Manduca (Zitnan et al., 1995). Even if overexpression of *FMRFa* in all neurosecretory cells in the *wit* rescue experiment could be due to a novel role for FMRFamide in ecdysis regulation in the CNS, it seems unlikely that this would apply in the wild-type condition, in which the Wit pathway is only regulating systemic FMRFamide. For the systemic peptide to have any central effect it would have to cross the blood-brain barrier that sheaths the nervous system (Carlson et al., 2000; Kretzschmar and Pflugfelder, 2002). Although this is possible, it seems more likely that the systemic delivery system has specifically evolved to function only on peripheral tissues and not the CNS itself. Effects of FMRFs in the CNS seem more likely to be mediated by the other neuroendocrine cells that are not affected in *wit* mutants. It is apparent that a complete dissection of the roles of systemic FMRFamide verse central FMRFs in synaptic transmission and eclosion will need to await the isolation of *FMRFa* mutants.

How Wit signaling regulates *FMRFa* expression is not clear. As Smads are well known to act as transcriptional co-activators or co-repressors (Attisano and Wrana, 2002; Moustakas et al., 2001) the simplest explanation is that Mad directly regulates activation of *FMRFa* transcription, perhaps by forming a complex with Ap. However, other indirect mechanisms are also possible and this issue will only be resolved once the *FMRFa* promoter is fully characterized. It is also not clear whether Gbb is the only ligand that regulates *FMRFa* expression through Wit. In some developmental contexts, such as wing imaginal disc patterning, Gbb acts in combination with Dpp, another Bmp-type ligand (Haerry et al., 1998; Khalsa et al., 1998). We do not see any expression of *dpp* in the NHO. However, it could be that one of the as yet uncharacterized ligands, Maverick or Myoglianin, could be a partner with Gbb in regulating *FMRFa* expression. Conversely, it seems clear that regulating the peptidergic phenotype of the six Tv neurons is not the only role of Gbb signaling. There are hundreds of neurons that receive Bmp signaling as indicated by P-Mad nuclear localization Most of them appear to be motoneurons, which require Wit/Gbb

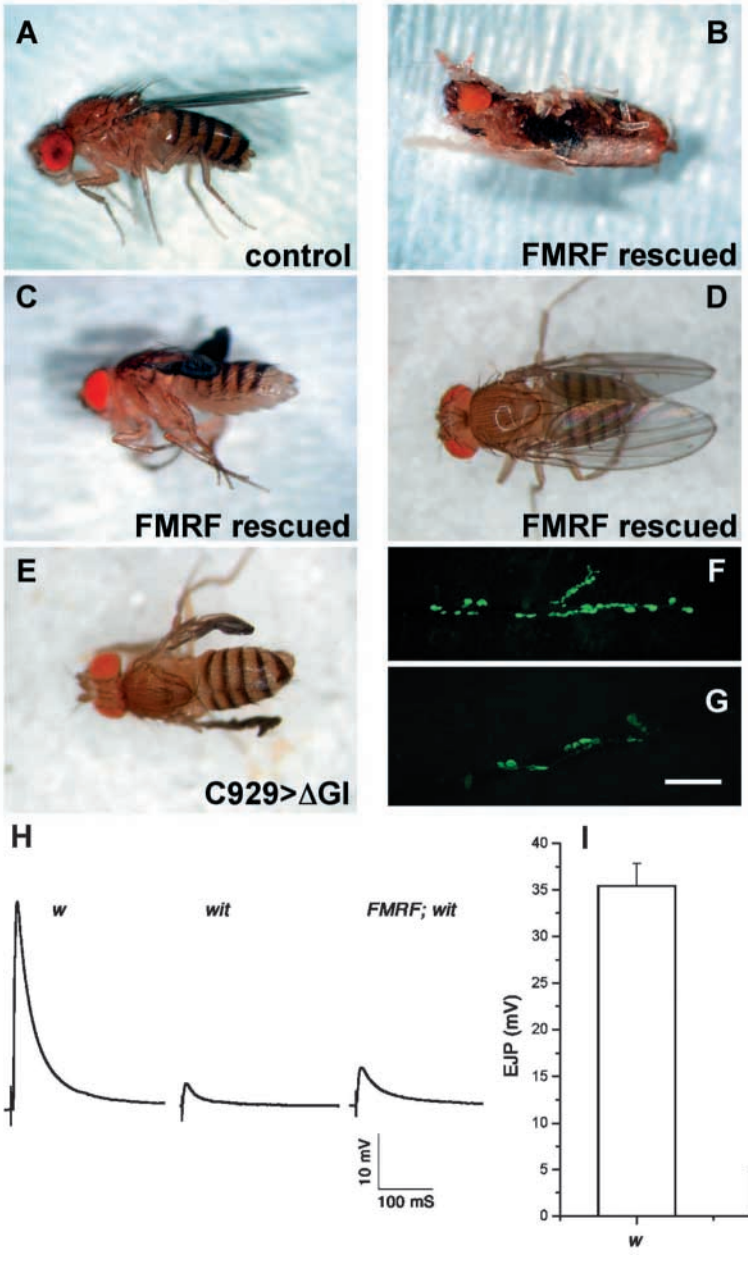


Fig. 9. Expression of FMRFa in neuroendocrine cells enables *wit* mutants to eclose without rescuing the primary defects in synaptic transmission or synapse morphology. (A) Wild-type Oregon R. (B-D) Partial rescue of *wit* adults in which FMRFa is expressed in neuroendocrine cells using the C929 driver (*w*; *P{UAS>FMRFa, w*$^{+}$*}/P{C929>Gal4, w*$^{+}$*}*; *wit*^{A12}, *st*/*wit*^{B11}, *st)*. (B) Partial eclosion, (C) full eclosion but no wing inflation, and (D) full eclosion and full wing inflation. (E) Lack of wing inflation in adult expressing ΔGl in neuroendocrine cells. (F) Wild-type synapse at muscles 6/7 visualized by anti-Csp staining. (G) Small synapse at muscles 6/7 of *wit*^{A12}/*wit*^{B11} animals expressing FMRFa with the C929 driver. (H) Representative traces of evoked junctional potentials of wild type, *wit* mutant and *wit* mutant expressing FMRFa. (I) Histogram representation of 5 recordings. Scale bar: 20 μm.

signaling to achieve proper synaptic growth but not to specify their neurotransmitter phenotype (Aberle, 2002; Marqués, 2002). Given that Smads act as co-transcriptional regulators, the fact that the same signal (nuclear translocation of P-Mad) results in different phenotypic outcomes in different neurons can probably be ascribed to the presence of a different set of transcription factors available in each cell type. The Tv neurons receiving the Bmp signal express *apterous*, a transcription factor required in those cells for *FMRFa* transcription (Benveniste, 1998), and maybe other factors that are required, in addition to the Wit signal, to activate *FMRFa*.

Another important issue to resolve is whether Gbb is constitutively released from the NHO, or is synthesized and released as part of a feedback mechanism to modulate muscle contractions. It might be that efficient muscle contraction under normal conditions requires a constant level of FaRPs that are produced in response to a constitutive Gbb signal. Alternatively, Gbb production or release might be regulated by a sensing mechanism that would activate the pathway in response to an increased demand for FaRPs, owing to increased locomotor activity or other stimuli, such as compensating for a synaptic developmental defect. We have recently found that muscle-derived Gbb acts through neuronal Wit to convey a retrograde signal essential for NMJ synapse growth and maturation (McCabe et al., 2003). In that context, it appears that the role of Bmp signaling is to coordinate muscle growth with synapse maturation to ensure proper synaptic efficiency. Thus, our combined results indicate that the Wit/Gbb pathway acts as a two-step regulator of NMJ function. First, there is a developmental role in which Wit signaling is required for proper synaptic growth during larval development (Aberle et al., 2002; Marqués, 2002; McCabe et al., 2003). Second, Wit signaling is required to achieve the neuromodulatory effect of circulating FaRPs that are required for optimal synaptic transmission. Lack of either one of these inputs probably results in a substantial decrease of the EJCs. These two examples suggest that the Gbb/Wit pathway is of general importance in neural retrograde signaling and we speculate that it may be used in the nervous system for other as yet uncharacterized developmental and physiological purposes.

Retrograde signaling by target-derived Gbb

Tv neurosecretory cells form part of a cluster of four *apterous*-expressing neurons on each side of the three thoracic ganglia. The axons of the Tv neurons extend proximally and dorsally to join the contralateral axon, and form a median nerve that swells and arborizes onto a group of neurons and glial cells that constitute the neurohemal organ (Benveniste et al., 1998). In *wit* mutants, these structures develop normally, but the Tv neuron fail to activate *FMRFa* transcription. Using the Gal4/UAS system we narrowed Wit's requirement for *FMRFa* expression to the Tv neurons. As these neurons accumulate nuclear P-Mad, the results strongly suggest that Wit is required in the Tv neurons themselves, as opposed to forming part of an indirect signal relay mechanism. It appears likely that the source of Gbb in this signaling system is the NHO, as *gbb* is expressed in the NHO and replenishing Gbb in the NHO of *gbb* mutants rescues *FMRFa* expression in the Tv neurons. These experiments do not exclude the possibility that signaling might occur at the cell soma of the Tv neurons in vivo or that the source of the diffusible ligand could be a different tissue under physiological conditions. However, the dependence of nuclear P-Mad accumulation and *FMRFa* expression in Tv neurons on Dynein-mediated retrograde transport strongly suggests that signaling is taking place at the Tv axon terminal. This dependency on Dynein motors is not a general requirement for *FMRFa* expression in all neurons because subesophageal ganglion neurons are not affected by overexpression of dominant-negative Glued or Dynamitin. Nor is the consequence of disrupting this motor likely to exert its effect at the level of P-Mad translocation to the nucleus, as nuclear accumulation of P-Mad in epithelial and mesodermal cells is not effected by retrograde transport disruption. Only in the nervous system is P-Mad accumulation specifically affected (McCabe et al., 2003), consistent with a role for a retrograde transport mechanism in moving some component of this signaling pathway from the synapse to the nucleus.

It is interesting to note that others have recently demonstrated that misexpressing ΔGl in the nervous system results in a decrease of quantal content and the number of synaptic boutons at the larval NMJ (Eaton et al., 2002). These phenotypes are strikingly similar to *wit* mutants, and are consistent with the notion that disruption of retrograde transport prevents Bmp signals from reaching the nucleus. These investigators suggested that the role of Dynactin is to maintain the presynaptic microtubule cytoskeleton thus contributing to synapse stability. However, our data and experiments described elsewhere (McCabe et al., 2003) indicate that failure to traffic a Gbb signal from axon to nucleus also likely contributes to the synaptic defects exhibited by overexpression of ΔGl in motoneurons.

The block of P-Mad accumulation and *FMRFa* expression upon disruption of retrograde transport suggests that one of the components of the signaling pathway has to be transported along the microtubules from the axon arborizations to the neuron cell body. The normal nuclear accumulation of P-Mad in epithelial and mesodermal cells in which Dynactin function has been disrupted makes it unlikely that the signaling block is at the level of receptor internalization (Penheiter et al., 2002) or translocation of P-Mad from cytoplasm to the nucleus. One possibility is that Mad itself could be phosphorylated at the axon terminal and then transported, either alone or in conjunction with other components, to the nucleus. However, a second possibility is that an activated receptor complex is transported back to the cell body. In the case of Neurotrophins, which constitute the best studied retrograde signaling pathway, it appears that ligand/activated receptor complexes are internalized through a clathrin mediated mechanism, and this signaling endosome is then routed to its appropriate cellular compartment through Dynein mediated retrograde traffic (Ginty and Segal, 2002; Miller and Kaplan, 2001; Miller and Kaplan, 2002; Yano et al., 2001). In the case of Wit, the receptor complex in the signaling endosome would probably contain the activated type I receptors Tkv and Sax (Hayes et al., 2002), and perhaps also Gbb. Mad phosphorylation could occur in the cytoplasm after retrograde transport of the signaling endosome. The dependence of Bmp signaling in the CNS on receptor internalization is supported by analysis of mutations in the *spinster* gene. Spinster is a component of the late endosomes/lysosomes, and mutations in this gene result in enhanced Wit signaling and synaptic overgrowth, perhaps

because of faulty downregulation of signaling endosomes (Sanyal and Ramaswami, 2002; Sweeney and Davis, 2002).

TGFβs as global regulators of synaptic growth and plasticity

Proper synaptic transmission requires formation, maintenance and pruning or strengthening of specific synapses in response to developmental or environmental stimuli. Our observations that Bmps control synaptic activity by regulating synapse growth as well as the expression of the neuromodulatory FaRPs, is particularly intriguing in light of another recent report that the *Drosophila* Activin pathway also regulates synapse function by stimulating pruning of mushroom body synapses during the larval to pupal transition (Zheng et al., 2003). Whether this involves a retrograde Activin signal is not clear, but it emphasizes the notion that this family of growth factors appears to have been recruited multiple times during evolution to regulate different aspects of synaptic function in invertebrates. Consistent with this view are previous reports that in *Aplysia*, TGFβ can induce long-term synaptic facilitation (Chin et al., 1999; Zhang et al., 1997). Given that various TGFβ-type ligands and their receptors are expressed in specific regions of the adult rodent brain (Charytoniuk et al., 2000), it will be interesting to determine if these proteins also participate in modulating synaptic function in mammals, particularly in regard to long-term memory and learning.

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Note added in proof

While this paper was under review, a similar set of findings were described by Allan et al. (Allan et al., 2003).

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 signalling and a combinatorial transcription factor code. *Cell* **113**, 73-86.

Askwith, C. C., Cheng, C., Ikuma, M., Benson, C., Price, M. P. and Welsh, M. J. (2000). Neuropeptide FF and FMRFamide potentiate acid-evoked currents from sensory neurons and proton-gated DEG/ENaC channels. *Neuron* **26**, 133-141.

Attisano, L. and Wrana, J. L. (2002). Signal transduction by the TGF-beta superfamily. *Science* **296**, 1646-1647.

Benveniste, R. J. and Taghert, P. H. (1999). Cell type-specific regulatory sequences control expression of the Drosophila FMRF-NH2 neuropeptide gene. *J. Neurobiol.* **38**, 507-520.

Benveniste, R. J., Thor, S., Thomas, J. B. and Taghert, P. H. (1998). Cell type-specific regulation of the Drosophila FMRF-NH2 neuropeptide gene by Apterous, a LIM homeodomain transcription factor. *Development* **125**, 4757-4765.

Brand, A. and Perrimon, N. (1993). Targetd gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brummel, T., Abdollah, S., Haerry, T. E., Shimell, M. J., Merriam, J., Raftery, L., Wrana, J. L. and O'Connor, M. B. (1999). The Drosophila activin receptor baboon signals through dSmad2 and controls cell proliferation but not patterning during larval development. *Genes Dev.* **13**, 98-111.

Brummel, T. J., Twombly, V., Marques, G., Wrana, J. L., Newfeld, S. J., Attisano, L., Massague, J., O'Connor, M. B. and Gelbart, W. M. (1994). Characterization and relationship of Dpp receptors encoded by the saxophone and thick veins genes in Drosophila. *Cell* **78**, 251-261.

Carlson, S. D., Juang, J. L., Hilgers, S. L. and Garment, M. B. (2000). Blood barriers of the insect. *Annu. Rev. Entomol.* **45**, 151-174.

Cazzamali, G. and Grimmelikhuijzen, C. J. (2002). Molecular cloning and functional expression of the first insect FMRFamide receptor. *Proc. Natl. Acad. Sci. USA* **99**, 12073-12078.

Charytoniuk, D. A., Traiffort, E., Pinard, E., Issertial, O., Seylaz, J. and Ruat, M. (2000). Distribution of bone morphogenetic protein and bone morphogenetic protein receptor transcripts in the rodent nervous system and up- regulation of bone morphogenetic protein receptor type II in hippocampal dentate gyrus in a rat model of global cerebral ischemia. *Neuroscience* **100**, 33-43.

Chiba, O., Sasaki, K., Higuchi, H. and Takashima, K. (1992). G-protein mediating the slow depolarization induced by FMRFamide in the ganglion cells of Aplysia. *Neurosci. Res.* **15**, 255-264.

Chin, J., Angers, A., Cleary, L. J., Eskin, A. and Byrne, J. H. (1999). TGF-beta1 in Aplysia: role in long-term changes in the excitability of sensory neurons and distribution of TbetaR-II-like immunoreactivity. *Learn. Mem.* **6**, 317-330.

Clark, I. E., Dobi, K. C., Duchow, H. K., Vlasak, A. N. and Gavis, E. R. (2002). A common translational control mechanism functions in axial patterning and neuroendocrine signaling in Drosophila. *Development* **129**, 3325-3334.

Cottrell, G. A. (1997). The first peptide-gated ion channel. *J. Exp. Biol.* **200**, 2377-2386.

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.

Duncan, J. E. and Warrior, R. (2002). The cytoplasmic Dynein and Kinesin motors have interdependent roles in patterning the Drosophila oocyte. *Curr. Biol.* **12**, 1982-1991.

Eaton, B. A., Fetter, R. D. and Davis, G. W. (2002). Dynactin is necessary for synapse stabilization. *Neuron* **34**, 729-741.

Echeverri, C. J., Paschal, B. M., Vaughan, K. T. and Vallee, R. B. (1996). Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J. Cell Biol.* **132**, 617-633.

Eckley, D. M., Gill, S. R., Melkonian, K. A., Bingham, J. B., Goodson, H. V., Heuser, J. E. and Schroer, T. A. (1999). Analysis of dynactin subcomplexes reveals a novel actin-related protein associated with the arp1 minifilament pointed end. *J. Cell Biol.* **147**, 307-320.

Fann, M. J. and Patterson, P. H. (1994). Depolarization differentially regulates the effects of bone morphogenetic protein (BMP)-2, BMP-6, and activin A on sympathetic neuronal phenotype. *J. Neurochem.* **63**, 2074-2079.

Feng, X. H., Filvaroff, E. H. and Derynck, R. (1995). Transforming growth factor-beta (TGF-beta)-induced down-regulation of cyclin A expression requires a functional TGF-beta receptor complex. Characterization of chimeric and truncated type I and type II receptors. *J. Biol. Chem.* **270**, 24237-24245.

Fuse, M. and Truman, J. W. (2002). Modulation of ecdysis in the moth Manduca sexta: the roles of the suboesophageal and thoracic ganglia. *J. Exp. Biol.* **205**, 1047-1058.

Galter, D., Bottner, M., Krieglstein, K., Schomig, E. and Unsicker, K. (1999). Differential regulation of distinct phenotypic features of serotonergic neurons by bone morphogenetic proteins. *Eur. J. Neurosci.* **11**, 2444-2452.

Gayton, R. J. (1982). Mammalian neuronal actions of FMRFamide and the structurally related opioid Met-enkephalin-Arg6-Phe7. *Nature* **298**, 275-276.

Geary, T. G., Marks, N. J., Maule, A. G., Bowman, J. W., Alexander-Bowman, S. J., Day, T. A., Larsen, M. J., Kubiak, T. M., Davis, J. P. and Thompson, D. P. (1999). Pharmacology of FMRFamide-related peptides in helminths. *Ann. New York Acad. Sci.* **897**, 212-227.

Ginty, D. D. and Segal, R. A. (2002). Retrograde neurotrophin signaling: Trk-ing along the axon. *Curr. Opin. Neurobiol.* **12**, 268-274.

Grimmelikhuijzen, C. J., Graff, D. and McFarlane, I. D. (1989). Neurones and neuropeptides in coelenterates. *Arch. Histol. Cytol.* **52**, 265-278.

Guan, Z., Giustetto, M., Lomvardas, S., Kim, J. H., Miniaci, M. C., Schwartz, J. H., Thanos, D. and Kandel, E. R. (2002). Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. *Cell* **111**, 483-493.

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.

Hattori, A., Katayama, M., Iwasaki, S., Ishii, K., Tsujimoto, M. and Kohno, M. (1999). Bone morphogenetic protein-2 promotes survival and differentiation of striatal GABAergic neurons in the absence of glial cell proliferation. *J. Neurochem.* **72**, 2264-2271.

Hayes, S., Chawla, A. and Corvera, S. (2002). TGF beta receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2. *J. Cell Biol.* **158**, 1239-1249.

Hewes, R. S., Snowdeal, E. C., 3rd, Saitoe, M. and Taghert, P. H. (1998). Functional redundancy of FMRFamide-related peptides at the Drosophila larval neuromuscular junction. *J. Neurosci.* **18**, 7138-7151.

Hinuma, S., Shintani, Y., Fukusumi, S., Iijima, N., Matsumoto, Y., Hosoya, M., Fujii, R., Watanabe, T., Kikuchi, K., Terao, Y. et al. (2000). New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. *Nat. Cell Biol.* **2**, 703-708.

Jan, L. Y. and Jan, Y. N. (1976). L-glutamate as an excitatory transmitter at the Drosophila larval neuromuscular junction. *J. Physiol.* **262**, 215-236.

Januschke, J., Gervais, L., Dass, S., Kaltschmidt, J. A., Lopez-Schier, H., Johnston, D. S., Brand, A. H., Roth, S. and Guichet, A. (2002). Polar transport in the Drosophila oocyte requires Dynein and Kinesin I cooperation. *Curr. Biol.* **12**, 1971-1981.

Jiang, N., Kolhekar, A. S., Jacobs, P. S., Mains, R. E., Eipper, B. A. and Taghert, P. H. (2000). PHM is required for normal developmental transitions and for biosynthesis of secretory peptides in Drosophila. *Dev. Biol.* **226**, 118-136.

Jordan, J., Bottner, M., Schluesener, H. J., Unsicker, K. and Krieglstein, K. (1997). Bone morphogenetic proteins: neurotrophic roles for midbrain dopaminergic neurons and implications of astroglial cells. *Eur. J. Neurosci.* **9**, 1699-1709.

Kalyani, A. J., Piper, D., Mujtaba, T., Lucero, M. T. and Rao, M. S. (1998). Spinal cord neuronal precursors generate multiple neuronal phenotypes in culture. *J. Neurosci.* **18**, 7856-7868.

Kavaliers, M. (1990). Inhibitory influences of mammalian FMRFamide (Phe-Met-Arg-Phe-amide)-related peptides on nociception and morphine- and stress-induced analgesia in mice. *Neurosci. Lett.* **115**, 307-312.

Kavaliers, M. and Yang, H. Y. (1991). Effects of mammalian FMRF-NH2-related peptides and IgG from antiserum against them on aggression and defeat-induced analgesia in mice. *Peptides* **12**, 235-239.

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.

Kolhekar, A. S., Roberts, M. S., Jiang, N., Johnson, R. C., Mains, R. E., Eipper, B. A. and Taghert, P. H. (1997). Neuropeptide amidation in Drosophila: separate genes encode the two enzymes catalyzing amidation. *J Neurosci* **17**, 1363-1376.

Kretzschmar, D. and Pflugfelder, G. O. (2002). Glia in development, function, and neurodegeneration of the adult insect brain. *Brain Res Bull* **57**, 121-131.

Lange, A. B. and Orchard, I. (1998). The effects of SchistoFLRFamide on contractions of locust midgut. *Peptides* **19**, 459-467.

Lingueglia, E., Champigny, G., Lazdunski, M. and Barbry, P. (1995). Cloning of the amiloride-sensitive FMRFamide peptide-gated sodium channel. *Nature* **378**, 730-733.

Lo, L., Tiveron, M. C. and Anderson, D. J. (1998). MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. *Development* **125**, 609-620.

Lopez-Coviella, I., Berse, B., Krauss, R., Thies, R. S. and Blusztajn, J. K. (2000). Induction and maintenance of the neuronal cholinergic phenotype in the central nervous system by BMP-9. *Science* **289**, 313-316.

Mackey, S. L., Glanzman, D. L., Small, S. A., Dyke, A. M., Kandel, E. R. and Hawkins, R. D. (1987). Tail shock produces inhibition as well as sensitization of the siphon-withdrawal reflex of Aplysia: possible behavioral role for presynaptic inhibition mediated by the peptide Phe-Met-Arg-Phe-NH2. *Proc. Natl. Acad. Sci. USA* **84**, 8730-8734.

Marqués, 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.

Martin, M., Iyadurai, S. J., Gassman, A., Gindhart, J. G., Jr, Hays, T. S. and Saxton, W. M. (1999). Cytoplasmic dynein, the dynactin complex, and kinesin are interdependent and essential for fast axonal transport. *Mol. Biol. Cell* **10**, 3717-3728.

McCabe, B., Marqués, 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

McCormick, J., Lim, I. and Nichols, R. (1999). Neuropeptide precursor processing detected by triple immunolabeling. *Cell Tissue Res.* **297**, 197-202.

McNabb, S. L., Baker, J. D., Agapite, J., Steller, H., Riddiford, L. M. and Truman, J. W. (1997). Disruption of a behavioral sequence by targeted death of peptidergic neurons in Drosophila. *Neuron* **19**, 813-823.

Mehler, M. F., Mabie, P. C., Zhang, D. and Kessler, J. A. (1997). Bone morphogenetic proteins in the nervous system. *Trends Neurosci.* **20**, 309-317.

Merte, J. and Nichols, R. (2002). Drosophila melanogaster FMRFamide-containing peptides: redundant or diverse functions? *Peptides* **23**, 209-220.

Miller, F. D. and Kaplan, D. R. (2001). On Trk for retrograde signaling. *Neuron* **32**, 767-770.

Miller, F. D. and Kaplan, D. R. (2002). Neurobiology. TRK makes the retrograde. *Science* **295**, 1471-1473.

Moustakas, A., Souchelnytskyi, S. and Heldin, C. H. (2001). Smad regulation in TGF-beta signal transduction. *J. Cell Sci.* **114**, 4359-4369.

Nachman, R. J., Holman, G. M., Haddon, W. F. and Ling, N. (1986). Leucosulfakinin, a sulfated insect neuropeptide with homology to gastrin and cholecystokinin. *Science* **234**, 71-73.

Nambu, J. R., Murphy-Erdosh, C., Andrews, P. C., Feistner, G. J. and Scheller, R. H. (1988). Isolation and characterization of a Drosophila neuropeptide gene. *Neuron* **1**, 55-61.

Nguyen, M., Park, S., Marques, G. and Arora, K. (1998). Interpretation of a BMP activity gradient in Drosophila embryos depends on synergistic signaling by two type I receptors, SAX and TKV. *Cell* **95**, 495-506.

Nichols, R. (1992). Isolation and structural characterization of Drosophila TDVDHVFLRFamide and FMRFamide-containing neural peptides. *J. Mol. Neurosci.* **3**, 213-218.

Nichols, R. (2003). Signaling pathways and physiological functions of Drosophila melanogaster FMRFamide-related peptides. *Annu. Rev. Entomol.* **48**, 485-503.

Nishimura, M., Ohtsuka, K., Takahashi, H. and Yoshimura, M. (2000). Role of FMRFamide-activated brain sodium channel in salt-sensitive hypertension. *Hypertension* **35**, 443-450.

Nolan, K. M., Sarafi-Reinach, T. R., Horne, J. G., Saffer, A. M. and Sengupta, P. (2002). The DAF-7 TGF-beta signaling pathway regulates chemosensory receptor gene expression in C. elegans. *Genes Dev.* **16**, 3061-3073.

O'Brien, M. A., Schneider, L. E. and Taghert, P. H. (1991). In situ hybridization analysis of the FMRFamide neuropeptide gene in Drosophila. II. Constancy in the cellular pattern of expression during metamorphosis. *J. Comp. Neurol.* **304**, 623-638.

Penheiter, S. G., Mitchell, H., Garamszegi, N., Edens, M., Dore, J. J., Jr and Leof, E. B. (2002). Internalization-dependent and -independent requirements for transforming growth factor beta receptor signaling via the Smad pathway. *Mol. Cell Biol.* **22**, 4750-4759.

Price, D. and Greenberg, M. (1977). Purification and characterization of cardioexcitatory neuropeptide from the central ganglia of a bivalve mollusc. *Prep. Biochem.* **7**, 261-281.

Rathmayer, W., Djokaj, S., Gaydukov, A. and Kreissl, S. (2002). The neuromuscular junctions of the slow and the fast excitatory axon in the closer of the crab Eriphia spinifrons are endowed with different Ca2+ channel types and allow neuron-specific modulation of transmitter release by two neuropeptides. *J. Neurosci.* **22**, 708-717.

Rawson, J., Lee, M. and Selleck, S. (2003). TGf-beta singaling in Drosophila regulates neuromuscular synapse size and function. *J. Neurobiol.* **55**, 134-150.

Reddy, S., Jin, P., Trimarchi, J., Caruccio, P., Phillis, R. and Murphey, R. K. (1997). Mutant molecular motors disrupt neural circuits in Drosophila. *J. Neurobiol.* **33**, 711-723.

Reiriz, J., Espejo, M., Ventura, F., Ambrosio, S. and Alberch, J. (1999). Bone morphogenetic protein-2 promotes dissociated effects on the number and differentiation of cultured ventral mesencephalic dopaminergic neurons. *J. Neurobiol.* **38**, 161-170.

Reissmann, E., Ernsberger, U., Francis-West, P. H., Rueger, D., Brickell, P. M. and Rohrer, H. (1996). Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons. *Development* **122**, 2079-2088.

Renn, S. C., Tomkinson, B. and Taghert, P. H. (1998). Characterization and cloning of tripeptidyl peptidase II from the fruit fly, Drosophila melanogaster. *J. Biol. Chem.* **273**, 19173-19182.

Robb, S. and Evans, P. (1994). The modulatory effect of Schisto FLRFamide on heart and Skeletal Muscle in the locust SCHISTOCERCA GREGARIA. *J. Exp. Biol.* **197**, 437-442.

Sanyal, S. and Ramaswami, M. (2002). Spinsters, synaptic defects, and amaurotic idiocy. *Neuron* **36**, 335-338.

Schneider, C., Wicht, H., Enderich, J., Wegner, M. and Rohrer, H. (1999). Bone morphogenetic proteins are required in vivo for the generation of sympathetic neurons. *Neuron* **24**, 861-870.

Schneider, L. E. and Taghert, P. H. (1988). Isolation and characterization of a Drosophila gene that encodes multiple neuropeptides related to Phe-Met-Arg-Phe-NH2 (FMRFamide). *Proc. Natl. Acad. Sci. USA* **85**, 1993-1997.

Schneider, L. E. and Taghert, P. H. (1990). Organization and expression of the Drosophila Phe-Met-Arg-Phe-NH2 neuropeptide gene. *J. Biol. Chem.* **265**, 6890-6895.

Schneider, L. E., O'Brien, M. A. and Taghert, P. H. (1991). In situ hybridization analysis of the FMRFamide neuropeptide gene in Drosophila. I. Restricted expression in embryonic and larval stages. *J. Comp. Neurol.* **304**, 608-622.

Schneider, L. E., Roberts, M. S. and Taghert, P. H. (1993a). Cell type-specific transcriptional regulation of the Drosophila FMRFamide neuropeptide gene. *Neuron* **10**, 279-291.

Schneider, L. E., Sun, E. T., Garland, D. J. and Taghert, P. H. (1993b). An immunocytochemical study of the FMRFamide neuropeptide gene products in Drosophila. *J. Comp. Neurol.* **337**, 446-460.

Small, S. A., Kandel, E. R. and Hawkins, R. D. (1989). Activity-dependent enhancement of presynaptic inhibition in Aplysia sensory neurons. *Science* **243**, 1603-1606.

Sweeney, S. T. and Davis, G. W. (2002). Unrestricted synaptic growth in spinster-a late endosomal protein implicated in TGF-beta-mediated synaptic growth regulation. *Neuron* **36**, 403-416.

Taghert, P. H., Hewes, R. S., Park, J. H., O'Brien, M. A., Han, M. and Peck, M. E. (2001). Multiple amidated neuropeptides are required for normal circadian locomotor rhythms in Drosophila. *J. Neurosci.* **21**, 6673-6686.

Tang, J., Yang, H. Y. and Costa, E. (1984). Inhibition of spontaneous and opiate-modified nociception by an endogenous neuropeptide with Phe-Met-Arg-Phe-NH2-like immunoreactivity. *Proc. Natl. Acad. Sci. USA* **81**, 5002-5005.

Twombly, V., Blackman, R. K., Jin, H., Graff, J. M., Padgett, R. W. and Gelbart, W. M. (1996). The TGF-beta signaling pathway is essential for Drosophila oogenesis. *Development* **122**, 1555-1565.

van Tol-Steye, H., Lodder, J. C., Mansvelder, H. D., Planta, R. J., van Heerikhuizen, H. and Kits, K. S. (1999). Roles of G-protein beta gamma, arachidonic acid, and phosphorylation inconvergent activation of an S-like potassium conductance by dopamine, Ala-Pro-Gly-Trp-NH2, and Phe-Met-Arg-Phe-NH2. *J. Neurosci.* **19**, 3739-3751.

Varley, J. E. and Maxwell, G. D. (1996). BMP-2 and BMP-4, but not BMP-6, increase the number of adrenergic cells which develop in quail trunk neural crest cultures. *Exp. Neurol.* **140**, 84-94.

Volterra, A. and Siegelbaum, S. A. (1988). Role of two different guanine nucleotide-binding proteins in the antagonistic modulation of the S-type K+ channel by cAMP and arachidonic acid metabolites in Aplysia sensory neurons. *Proc. Natl. Acad. Sci. USA* **85**, 7810-7814.

Wang, Z., Orchard, I. and Lange, A. B. (1994). Identification and characterization of two receptors for SchistoFLRFamide on locust oviduct. *Peptides* **15**, 875-882.

Wang, Z., Orchard, I. and Lange, A. B. (1995a). Binding affinity and physiological activity of some HVFLRFamide analogues on the oviducts of the locust, Locusta migratoria. *Regul. Pept.* **57**, 339-346.

Wang, Z., Orchard, I., Lange, A. B. and Chen, X. (1995b). Mode of action of an inhibitory neuropeptide SchistoFLRFamide on the locust oviduct visceral muscle. *Neuropeptides* **28**, 147-155.

Wharton, K. A., Thomsen, G. H. and Gelbart, W. M. (1991). Drosophila 60A gene, another transforming growth factor beta family member, is closely related to human bone morphogenetic proteins. *Proc. Natl. Acad. Sci. USA* **88**, 9214-9218.

Wharton, K. A., Cook, J. M., Torres-Schumann, S., de Castro, K., Borod, E. and Phillips, D. A. (1999). Genetic analysis of the bone morphogenetic protein-related gene, gbb, identifies multiple requirements during Drosophila development. *Genetics* **152**, 629-640.

Yang, H. Y., Fratta, W., Majane, E. A. and Costa, E. (1985). Isolation, sequencing, synthesis, and pharmacological characterization of two brain neuropeptides that modulate the action of morphine. *Proc. Natl. Acad. Sci. USA* **82**, 7757-7761.

Yano, H., Lee, F. S., Kong, H., Chuang, J., Arevalo, J., Perez, P., Sung, C. and Chao, M. V. (2001). Association of Trk neurotrophin receptors with components of the cytoplasmic dynein motor. *J. Neurosci.* **21**, RC125.

Zhang, B., Koh, Y. H., Beckstead, R. B., Budnik, V., Ganetzky, B. and Bellen, H. J. (1998). Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. *Neuron* **21**, 1465-1475.

Zhang, F., Endo, S., Cleary, L. J., Eskin, A. and Byrne, J. H. (1997). Role of transforming growth factor-beta in long-term synaptic facilitation in Aplysia. *Science* **275**, 1318-1320.

Zheng, X., Wang, J., Haerry, T. E., Wu, A. Y., Martin, J., O'Connor, M. B., Lee, C. H. and Lee, T. (2003). Baboon/Smad2-mediated TGF-β signaling activates ecdysone receptor B1 expression during neuronal remodeling in the *Drosophila* Brain. *Cell* (in press).

Zitnan, D., Kingan, T. G., Kramer, S. J. and Beckage, N. E. (1995). Accumulation of neuropeptides in the cerebral neurosecretory system of Manduca sexta larvae parasitized by the braconid wasp Cotesia congregata. *J. Comp. Neurol.* **356**, 83-100.

Zitnan, D., Zitnanova, I., Spalovska, I., Takac, P., Park, Y. and Adams, M. E. (2003). Conservation of ecdysis-triggering hormone signalling in insects. *J. Exp. Biol.* **206**, 1275-1289.