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## Synaptic transmission in neurons that express the *Drosophila* atypical soluble guanylyl cyclases, *Gyc-89Da* and *Gyc-89Db*, is necessary for the successful completion of larval and adult ecdysis

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### Abstract

Insect ecdysis is a precisely coordinated series of behavioral and hormonal events that occur at the end of each molt. A great deal is known about the hormonal events that underlie this process, although less is known concerning the neuronal circuitry involved. Here we identify two populations of neurons that are required for larval and adult ecdyses in the fruit fly, *Drosophila melanogaster*. These neurons were identified by using the upstream region of two genes that code for atypical soluble guanylyl cyclases to drive tetanus toxin in the neurons that express these cyclases to block their synaptic activity. Expression of tetanus toxin in neurons that express *Gyc-89Da* blocked adult eclosion whereas expression of tetanus toxin in neurons that express *Gyc-89Db* prevented the initiation of the first larval ecdysis. Expression of tetanus toxin in the *Gyc-89Da* neurons also resulted in about 50% lethality just prior to pupariation, however this was likely due to suffocation in the food as lethality was prevented by preventing the larvae from burrowing deep within the food. This result is consistent with our model that the atypical soluble guanylyl cyclases can act as molecular oxygen detectors. The expression pattern of these cyclases did not overlap with any of the neurons containing peptides known to regulate ecdysis and eclosion behaviors. By using the conditional expression of tetanus toxin we were also able to demonstrate that synaptic activity in the *Gyc-89Da* and *Gyc-89Db* neurons is required during early adult development for adult eclosion.

### Introduction

The intracellular messenger, cyclic 3'5' guanosine monophosphate (cGMP) regulates a wide variety of physiological processes in vertebrates and invertebrates (Lucas et al., 2000; Morton and Hudson, 2002). The synthesis of cGMP is catalyzed by guanylyl cyclases that can be classified as either receptor guanylyl cyclases, which are integral membrane proteins or soluble guanylyl cyclases, which are localized to the cytoplasm (Lucas et al., 2000). Soluble guanylyl cyclases can be further subdivided into conventional soluble guanylyl cyclases, which are the targets for the gaseous messenger, nitric oxide (NO) (Lucas et al., 2000) and the atypical soluble guanylyl cyclases that are poorly activated by NO (Morton, 2004a) and have been proposed to act as oxygen sensors (Gray et al., 2004). The *Drosophila* genome contains 5 genes that code for subunits of soluble guanylyl cyclases (sGCs) (Morton, 2004a). Two of these, *Gyca-99B* and *Gycβ-100B*, form the conventional NO-sensitive sGC and the other three genes code for atypical subunits that form oxygen-sensitive sGCs (Morton, 2004b). We have previously shown that the atypical sGC subunits, *Gyc-88E*, *Gyc-89Da* and *Gyc-89Db*, are expressed in a subset of central and chemosensory peripheral neurons (Langlais et al., 2004;

Morton et al., 2005a). Based on the expression pattern and biochemical properties of these subunits we predicted that the sGCs formed from the atypical subunits would be involved in olfaction, gustation and/or oxygen sensation (Vermehren et al., 2006).

In this manuscript we begin to examine the function of the neurons that express the atypical sGCs, *Gyc-89Da* and *Gyc-89Db*. Using the predicted promoter regions of these genes we have generated flies that express the yeast transcription factor, GAL4 (Brand and Perrimon, 1993) under the control of the promoters for each GC subunit. We then used these flies, to express the light chain of tetanus toxin (TNT) under the control of an upstream activation sequence (UAS) that is regulated by GAL4 to block synaptic transmission in the cells that express *Gyc-89Da* and *Gyc-89Db*. The results show that at least some of these neurons are required for the successful initiation and completion of larval and adult ecdysis.

## Methods

### Fly strains and genetics

A variety of GAL4 and UAS fly lines were kindly provided by the following labs: *Ap-GAL4* (*apterous-GAL4*) Paul Taghert, Washington University, St. Louis, USA; *CCAP-GAL4* (CCAP promoter) John Ewer, Cornell University, NY, USA; *dimmed-GAL4* (*dimmed*) Randy Hewes, University of Oklahoma, Norman, OK, USA; *EHups-GAL4* (eclosion hormone promoter driving GAL4) Sue McNabb, University of Washington, Seattle, USA; *UAS-TNTa* and *UAS-TNTi* lines (lines 1171 and 1178 that express the active and inactive forms of the light chain of tetanus toxin under UAS control) were provided by Cahir O'Kane & Sean Sweeney, University of Cambridge, England; *UAS-PDE5* line (bovine phosphodiesterase 5) Shireen Davies, University of Glasgow, Scotland. The *UAS-dsRed*, *tubp-GAL80ts* lines were obtained from the Bloomington Stock Center. All crosses were carried out using standard methods at 25° C (Greenspan, 1997) unless otherwise indicated.

### Cloning *Gyc-89Da* and *Gyc-89Db* promoter regions

The *Gyc-89Da* promoter (p89Da) lines were generated by cloning the 1.9 kb sequence 5' upstream of the translational start site of *Gyc-89Da* that included the 5' UTR and first intron. The 1931 bp PCR fragment was amplified from genomic DNA using the primers GGGTTCTTTTTGTAGAGAAA and GGTTTATGTTTCTGAAAAAATG and *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The PCR fragment was gel purified and cloned directly into the TOPO-TA vector (Invitrogen). The *Gyc-89Da* promoter fragment was then excised with *Bam*HI and *Not*I restriction enzymes (Invitrogen) and cloned into the p element vectors pPTGAL (Sharma et al., 2002) and GFP pGreen Pelican (Barolo et al., 2000). The *Gyc-89Db* promoter (p89Db) lines were generated in a similar manner by cloning the 3398 bp sequence 5' of *Gyc-89Db*, using the primers GCACCTGTGGCTCTCTTA and GATGGGGCAGGATGTGAA. The PCR fragment was then subcloned into the pPTGAL and GFP vectors. DNA for microinjection was prepared by ultracentrifugation using a CsCl gradient. About 200-400 *wy*<sup>1</sup>; embryos were co-injected with 0.2 µg/µl of helper plasmid (pπ25.7 Δ2-3 wc) and 1 µg/µl of either the GFP or GAL4 constructs in 0.85mM NaH<sub>2</sub>PO<sub>4</sub>, 9.15mM Na<sub>2</sub>HPO<sub>4</sub>, 5mM KCl buffer, and maintained at 18°C until eclosion. Eleven insertion lines were recovered for p89Da-GAL4, two for p89Da-GFP, four for p89Db-GAL4, and two for p89Db-GFP. Chromosomal locations of the P-element insertions were determined by standard crossing methods to a ubiquitous balancer line (*w*; *CyO/Kr<sup>1</sup>f-1*; *Tm6B,Tb<sup>+</sup>/D<sup>1</sup>*).

### In situ hybridization

In situ hybridization of embryos using digoxigenin-labeled probes was carried out as previously described (Langlais et al., 2004).

## Confocal microscopy

Tissue or whole larvae were mounted in aqueous mounting media (Gel Mount, Sigma, St. Louis, MO) and viewed on a Nikon Eclipse E800 fluorescence microscope equipped with argon and helium/neon lasers (Radiance 2100, Bio-Rad, Hercules, CA) to excite the GFP and dsRed proteins (emission filters 500-530 nm and 570-650 nm respectively). Usually, a stack of 15 sections was taken ( $z$ -axis step size, 2.5  $\mu\text{m}$ ) using Laser Sharp 2000 software (Bio-Rad) and image processing was carried out using *Image J* (<http://rsb.info.nih.gov/ij/>). Identification of neurons expressing GFP was made based on a combination of their morphologies and their relative position.

## Mortality/viability analysis

The mortality of developing animals expressing the active or inactive forms of TNT was determined as follows. To determine the hatching success rate, flies were allowed to lay eggs on standard fruit plates (Sullivan et al., 2000) overnight at 25° C. Embryos were collected and arranged in rows on a new fruit plate and allowed to hatch for 36 hours at 25° C. Hatched and un-hatched embryos were then counted. To examine the survival of 1<sup>st</sup> instar larvae to the end of the third instar stage and into the pupal stage, a known number of 1<sup>st</sup> instar larvae were placed in a standard food vial or on a custom food disc in a plastic 8.5cm Petri dish, with a few granules of dried yeast. The food disc was prepared by melting standard fly food, pouring it onto a large glass plate and compressing it between a second glass plate separated by 3mm spacers. When solidified, 5.6 cm diameter discs were then cut from this sheet. Larvae placed in vials or on discs were kept at 25° C until pupariation and counted. The developmental stage and location of any dead larvae were recorded. To examine defects during eclosion, developing adults were collected from vials at stage P15 and lined up on a strip of double-sided clear tape on a glass slide. Just prior to eclosion, the operculum was manually removed from each pupa and the pre-eclosion staging markers, (Kimura and Truman, 1990; McNabb et al., 1997) were observed through a dissecting microscope. At least 15 experimental and control animals from three separate crosses were examined for pre-eclosion defects.

## GAL80<sup>TS</sup> experiments

The temperature-sensitive inhibitor of GAL4, GAL80<sup>TS</sup> (Zeidler et al., 2004) was used in some experiments to control temporal expression of TNT. Using standard crossing schemes (Greenspan, 1997), a homozygous fly line was created that contained GAL80<sup>TS</sup> driven by the tubulin promoter (*tubP-GAL80<sup>TS</sup>*) on chromosome 2 and the *p89Da-GAL4* insertion on chromosome 3. These flies were then crossed with *UAS-TNTa*, *UAS-TNTi* or *UAS-DsRed* flies, resulting in offspring that contained *tubP-GAL4<sup>TS</sup>*, *p89Da-GAL4* and *UAS-TNTa*, *UAS-TNTi* or *UAS-DsRed*. These crosses were carried out at 18°C, the permissive temperature for GAL80<sup>TS</sup>. At 18°C, GAL80<sup>TS</sup> inhibits the action of GAL4. Experimental vials, as described in the results, were switched at specific stages to an incubator set at 30°C, the non-permissive temperature for GAL80<sup>TS</sup> (Zeidler et al., 2004) that allows GAL4 to function and activate transcription of UAS controlled transgenes.

## Results

### Expression of *Gyc-89Da* and *Gyc-89Db* sGC subunits in *Drosophila* larvae

Our previous studies using whole mount *in situ* hybridization labeling in embryos, showed that all three atypical sGC subunits, *Gyc-89Da*, *Gyc-89Db* and *Gyc-88E*, were expressed in a subset of peripheral and central neurons, (Langlais et al., 2004; Morton et al., 2005a). By examining the number and position of these cells we concluded that these three genes were co-expressed in many of the peripheral sensory neurons (Langlais et al., 2004; Morton et al., 2005a). To better define these co-expression patterns, we used upstream regions of each gene to drive

expression of GFP in transgenic flies (designated *p89Da-GFP* and *p89Db-GFP*). The portions of genomic DNA 5' to *Gyc-89Da* and *Gyc-89Db* (1.9 and 3.4 kb respectively) were sufficient to drive GFP expression in embryos in pattern that closely resembled that seen with *in situ* hybridization (Figure 1). By contrast, however, a 5 kb length of genomic DNA 5' to *Gyc-88E* was insufficient to drive expression of GFP in any cells. Peripheral neurons that expressed GFP in *p89Da-GFP* and *p89Db-GFP* larvae included a small subset of cells in the dorsal and terminal ganglia (Figure 1G-I), which innervate the olfactory and gustatory organs respectively (Stocker, 1994; Heimbeck et al., 1999), neurons innervating external sensilla along the lateral body wall (Figure 1D-F) and neurons that innervate the terminal sensory organs (Figure 1J-L) (Langlais et al., 2004; Morton et al., 2005a). These expression patterns matched closely to those seen using *in situ* hybridization in late embryos (Langlais et al., 2004) (Figure 1).

Most of the peripheral neurons that express GFP under the control of the *Gyc-89Da* and *Gyc-89Db* promoters have stereotyped positions and the relatively low number of cells that express GFP makes it possible to compare these preparations with the *in situ* hybridizations carried out in embryos. A comparison of the number of cells in each group labeled by each method is shown in Table 1. In general, there was a very good match in the number of cells in each group between the embryo *in situ* hybridization data and direct observation of GFP fluorescence driven by each of the sGC promoters. In the dorsal and terminal ganglia a small number of neurons (5 or less) were labeled with both *in situ* and direct visualization of GFP for both *Gyc-89Da* and *Gyc-89Db* (Table 1). Sensory neurons innervating external sensilla along the larval body wall can be broadly divided into three groups – dorsal (des cells), lateral (les cells) and ventral (ves cells) (Bodmer et al., 1987). In our previous studies using a combination of *in situ* hybridization and immunocytochemistry with a neuronal marker in embryos, we showed that *Gyc-89Da* and *Gyc-89Db* were expressed in a single les and a single ves cell in the thoracic segments and *Gyc-89Db* was expressed in a tracheal dendritic neuron (td cell) in the first two abdominal segments only (Langlais et al., 2004; Morton et al., 2005a). Direct visualization of GFP expression driven by the two promoters also labeled these cells. Two cells on each side of the larvae were observed in each thoracic segments of *p89Da-GFP* and *p89Db-GFP* larvae (Table 1). One of these cells was located in a more lateral position and the other in a more ventral position corresponding to the positions of the les and ves cells respectively. The td neuron that was identified in the first two abdominal segments of embryos using *in situ* hybridizations of *Gyc-89Db* (Langlais et al., 2004) was also observed in *p89Db-GFP* larvae with the exception that these cells were also seen in abdominal segment 3 (Table 1). In the terminal abdominal segments there are seven sensory organs on either side of the larvae (Stocker, 1994) and *in situ* hybridizations in embryos showed that a single neuron innervating 6 of these structures expressed *Gyc-89Da* and *Gyc-89Db* (Langlais et al., 2004; Morton et al., 2005a). Direct visualization of GFP in *p89Da-GFP* and *p89Db-GFP* larvae also revealed these neurons, and we were able to detect a single neuron in each of the seven sensory organs (Table 1). These experiments show that there is a very good match between expression of *Gyc-89Da* and *Gyc-89Db* determined by *in situ* hybridization and expression of GFP driven by portions of 5' genomic DNA.

Our previous studies (Langlais et al., 2004; Morton et al., 2005a) used cell counts to determine the overlap in the expression patterns of the three atypical sGC subunits. These experiments indicated that in the majority of peripheral cells *Gyc-89Da* and *Gyc-89Db* were co-expressed with *Gyc-88E* and suggested that in many cells all three subunits were co-expressed (Langlais et al., 2004; Morton et al., 2005a). To determine the co-expression patterns of *Gyc-89Da* and *Gyc-89Db* more accurately, we generated transgenic flies that expressed the yeast transcription factor, GAL4 under the control of the upstream regions of these genes (designated *p89Da-GAL4* and *p89Db-GAL4*) and crossed them with flies that expressed red fluorescent protein (dsRed) under UAS control. Examination of larvae from these crosses showed the same

numbers of peripheral neurons as seen with the *p89Da-GFP* and *p89Db-GFP* lines respectively (Table 1). We then generated larvae that contained three transgenes: *p89Da-GFP*, *p89Db-GAL4* and *UAS-dsRed* and examined the neurons under both green and red fluorescence. In larvae expressing *p89Da-GFP*, *p89Db-GAL4* and *UAS-dsRed* we consistently observed 4 neurons that expressed *Gyc-89Da* and 4 neurons that expressed *Gyc-89Db* in the dorsal ganglion, but none of these neurons co-expressed both subunits (Figure 2A). Similarly, in the terminal ganglion, 3-4 neurons expressed *Gyc-89Da* and 3-4 neurons expressed *Gyc-89Db*, but we never observed neurons in the terminal ganglion that expressed both subunits (Figure 2A). In addition to the neurons in the dorsal and terminal ganglia within the larval head, we consistently observed co-expression in two neurons that appeared to be part of the monoscolopidial chordotonal organs (Figure 2A), which lie adjacent to the terminal ganglia (Campos-Ortega and Hartenstein, 1997). Expression of *Gyc-89Db* alone was also seen in 2-3 multidendritic arborization neurons (Grueber et al., 2002) in the head region.

In contrast to the terminal and dorsal ganglia, co-expression of *Gyc-89Da* and *Gyc-89Db* was seen in peripheral neurons in the thoracic segments. Both cells in all three thoracic segments, co-expressed *Gyc-89Da* and *Gyc-89Db* (Figure 2B, Table 1). The td neuron in the abdominal segments, however, only expressed *Gyc-89Db* and did not show *Gyc-89Da* expression (Figure 2B, Table 1). In the caudal segments, *Gyc-89Da* and *Gyc-89Db* were co-expressed in neurons that innervate peg sensilla on the sensory cones (Figure 2C). There are 7 sensory cones on each side of the larvae, which have been described as possible chemosensory detectors (Stocker, 1994). Each sensory cone appears to be innervated by 1-2 neurons and only a single neuron in each cone co-expressed both subunits. A neurite was clearly visible extending to the peg sensilla at the tip of each cone (Figure 2C, inset).

*Gyc-89Da* and *Gyc-89Db* were also expressed in the larval CNS, but no co-expression of the two subunits was detected in central neurons (Figure 2D). Numerous cells were labeled in the brain lobes and ventral nerve cord, with a larger number of cells that expressed *Gyc-89Db* was observed compared to *Gyc-89Da*. The only cells where *Gyc-89Da* and *Gyc-89Db* were co-localized was in the ring gland (neurosecretory organ), where several intrinsic secretory cells of the corpora cardiaca expressed both subunits (data not shown).

### Neurotransmission in *Gyc-89Da* and *Gyc-89Db* neurons is required for complete development

To analyze the functions of the neurons that express *Gyc-89Da* and *Gyc-89Db* we blocked synaptic transmission in these cells by expressing the light chain of tetanus toxin (TNT) under UAS control (Sweeney et al., 1995). TNT abolishes transmitter release by cleaving the synaptic vesicle protein synaptobrevin (Schiavo et al. 1992; Link et al., 1992). Identification of behaviors that are disrupted when TNT is expressed in subsets of neurons in *Drosophila* provides a simple tool for examining the function of those neurons (Sweeney et al., 1995). When we expressed the active form of TNT (TNTa) in the neurons that express *Gyc-89Da* and *Gyc-89Db* the animals failed to develop into adult flies and died at three specific developmental stages associated with the molt (Figure 3). As a control we also used a fly line that expressed an inactive form of TNT (TNTi) under UAS control (Sweeney et al., 1995). About 90% of eggs generated from these crosses survived to adult (Figure 3). For those neurons that express *Gyc-89Da*, expression of TNT caused slightly reduced hatching rates ( $84.9 \pm 1.8\%$  compared to  $92.3 \pm 0.4\%$  for expression of TNTi,  $p < 0.05$ ). The majority of the hatchlings survived to the end of the 3<sup>rd</sup> instar and about 50% died at the beginning of metamorphosis, at about the time of pupariation. All of the animals that successfully pupated died just prior to adult eclosion (emergence from the pupal cuticle). By contrast, expression of TNT in the *Gyc-89Db*-expressing neurons caused all the animals to die at the first-second larval transition (Figure 3).

### Death of *p89Da-GAL4* × *UAS-TNTa* larvae during third instar possibly due to drowning

To determine if there were general behavioral deficits during larval stages, we tested the 3<sup>rd</sup> instar *p89Da-GAL4* × *UAS-TNTa* larvae for their general locomotion on 1% agarose plates (Osborne et al., 1997) and observed no significant differences between those expressing TNTa and TNTi in distance traveled ( $2.37 \pm 0.26$  and  $1.99 \pm 0.16$  cm/min, respectively; N=9 for each genotype, t-test  $p=0.2372$ ). Although locomotion and growth appeared normal, we found substantial numbers of late 3<sup>rd</sup> instar larvae that expressed TNTa in the *Gyc-89Da* neurons deeply buried in the food, whereas most of those that expressed TNTi had all crawled up the wall of the vial and formed puparia. We previously suggested that the *Drosophila* atypical sGCs act as neuronal O<sub>2</sub> sensors because they are activated by reduced O<sub>2</sub> concentrations (Morton, 2004b). In addition, a related sGC in *C. elegans* (GCY-35) has been shown to mediate O<sub>2</sub> sensitive behaviors (Gray et al., 2004). This suggested that larvae with inactive *Gyc-89Da* neurons might not be able to sense that they were deep in the food and suffocating. To prevent the TNTa-expressing animals from burying deeply into the food, Petri dishes containing a thin layer of food (~3mm thick) were used instead of the normal food vials (3 cm depth of food). Under these conditions, no significant differences in the number of animals that successfully pupated were observed between those expressing TNTa and TNTi (Figure 4).

### Neurons that express *Gyc-89Da* are necessary for expression of the adult eclosion motor program

Almost all ( $98.2 \pm 0.8$  %) of the *p89Da-GAL4* × *UAS-TNTa* larvae that successfully formed puparia failed to eclose as adult flies regardless of whether they were raised on thin discs of food or in normal vials. To examine more closely the causes of the failure to eclose, we examined animals during the final few hours before eclosion for the appearance of developmental markers that precede eclosion (Kimura and Truman, 1990). Animals were examined under a dissecting microscope and the opercula removed to facilitate detection of the markers. About 8 hours prior to eclosion the head of the developing adult is smooth, indicating that molting fluid is still present between the pupal and adult cuticle. About 3 hours prior to eclosion the head has a grainy appearance, which indicates that molting fluid absorption is complete. The tracheal system and the space between the adult and pupal cuticles fill with air about 1 hr before eclosion leading to a whitish appearance of the head. This is followed at about 40 min before eclosion by the extension of the ptilinum from the front of the head, which ruptures the thin pupal cuticle. All of these stages occurred in the proper order in *p89Da-GAL4* × *UAS-TNTa* and *p89Da-GAL4* × *UAS-TNTi* animals. Some animals were also removed from the pupal cases at the onset of tracheal air filling and examined under a microscope to determine the extent of tracheal air filling throughout the body, which appeared normal and was comparable to similarly staged control animals.

About 40 minutes after ptilinum extension, the *p89Da-GAL4* × *UAS-TNTi* control animals began inflating their heads and pushed forward, which enabled the animals to rupture and push open the operculum. After several strong head inflations (10-20 seconds), the *p89Da-GAL4* × *UAS-TNTi* control animals began the stereotyped abdominal, posterior directed peristalsis movements (eclosion behavior) leading to complete emergence from the puparium. The eclosion of these control animals is comparable to that of wild-type Canton-S animals (McNabb et al., 1997). The animals that expressed the active form of TNT (*p89Da-GAL4* × *UAS-TNTa*) also began head inflations about 40 minutes after ptilinum extension, but the magnitude of the inflations appeared much weaker. These weaker head inflations rarely succeeded in completely opening the operculum, when it was left intact. Several head inflation/deflations occurred and the animal did push forward noticeably, but the abdominal peristalsis component of the eclosion motor program was never initiated in these animals. Interestingly, head inflation/deflations occurred for up to 4 more hours. During this time, starting about an hour

after the first head inflations, the antennae occasionally twitched, and the abdomen sporadically contracted several times a minute, but these movements did not resemble the coordinated series of peristaltic contractions seen in control animals. Even when the operculum was removed just prior to the expected time of eclosion, the animals expressing TNTa in the *Gyc-89Da* neurons never succeeded in escaping from the puparium.

After wild-type flies have eclosed they undergo wing inflation and cuticle tanning (see Ewer and Reynolds, 2002). To determine if animals that expressed TNTa in the *Gyc-89Da* neurons completed these processes in the absence of eclosion, we removed them from their puparia and observed them for up to 2 hrs. These animals remained immobile, never showed any wing inflation and after 2 hrs still had un-tanned cuticle. By contrast, animals that expressed the TNTi inflated their wings and tanned their cuticles within 30 minutes of eclosion.

### Neurons that express *Gyc-89Db* are necessary for larval ecdysis

In contrast to the *p89Da-GAL4 x UAS-TNTa* progeny, the majority of which survived to the 3<sup>rd</sup> instar, when TNTa was expressed in the cells that express *Gyc-89Db*, all the larvae died before they reached the 2<sup>nd</sup> instar (Figure 3). Fewer of *p89Db-GAL4 x UAS-TNTa* progeny hatched compared to controls expressing TNTi ( $85.3 \pm 1.7\%$  vs.  $92.6 \pm 1.3\%$ ,  $p < 0.05$  Student's t test) and the newly hatched larvae expressing TNTa appeared to become increasingly sluggish after a day of feeding on the surface of the food. Although the larvae appeared morphologically normal they all failed to reach the 2<sup>nd</sup> larval instar. About 80% of the *p89Db-GAL4 x UAS-TNTa* larvae that hatched survived through the feeding phase and began the developmental events that lead to ecdysis ( $83.2 \pm 1.4\%$  compared to  $97.4 \pm 0.3\%$  TNTi controls,  $p < 0.05$  Student's t test) to the period just before ecdysis. Of the 16.8% of larvae that died during the feeding phase, most were found buried under the food (63% buried vs. 37% on top). This appeared to be similar to the 3<sup>rd</sup> instar larvae expressing TNTa in the *Gyc-89Da* neurons many of which appeared to drown in their food, possibly because they failed to detect that they had become anoxic.

There are several morphological markers that have been described that precede larval ecdysis in *Drosophila* (Park et al., 2002; Clark et al., 2004). About 1 hr prior to the 1<sup>st</sup> larval ecdysis the new mouth parts of the 2<sup>nd</sup> instar larvae become visible, a stage referred to as double vertical plates (dVP, Park et al., 2002). This is followed about 10 min later by the collapse of the old tracheal lining and almost immediately by the trachea filling with air. Shortly after tracheal air filling the pre-ecdysis and ecdysis behaviors commence (Park et al., 2002). Over 99% of the *p89Db-GAL4 x UAS-TNTa* larvae that survived past the 1<sup>st</sup> instar feeding phase were found with transparent fluid-filled double trachea and dVP. No animals were found with air-filled tracheae or that initiated pre-ecdysis or ecdysis peristaltic movements. We presume that the larvae found with dVP and transparent, fluid filled trachea likely died from suffocation as they were unable to air-fill their tracheae.

### *Gyc-89Da* and *Gyc-89Db* neurons do not contain EH or CCAP

The endocrinology underlying the initiation of ecdysis and eclosion in *Drosophila* has been extensively studied and is known to be regulated by the release and action of at least three peptide hormones: ecdysis triggering hormone (ETH), eclosion hormone (EH) and crustacean cardiac acceleratory peptide (CCAP) (McNabb et al. 1997; Park et al., 2002; Clark et al., 2004). Deletion of the gene encoding these peptides or elimination of the cells that secrete the peptide lead to similar phenotypes to those described above for expression of TNTa in the cells that express *Gyc-89Da* and *Gyc-89Db*. For example, deletion of the ETH gene leads to lethality at the first larval ecdysis with most of the animals dying after tracheal inflation and some animals showing some "ecdysis-like" movements although none initiated pre-ecdysis movements (Park et al., 2002). Killing the EH cells leads to larval death, with most of the larvae

dieing with fluid filled tracheae, although the timing of larval pre-ecdysis and ecdysis behaviors appeared unaffected (McNabb et al., 1997; Clark et al., 2004). Furthermore, EH cell knockouts led to disruption of adult eclosion, notably with a longer period between ptilinum extension and eclosion (McNabb et al., 1997). It is possible that the effects of TNTa expression in the *Gyc-89Da* and *Gyc-89Db* neurons were due to a failure of the release of some or all of these peptides. The simplest explanation for this would be if the TNTa was expressed in the peptidergic cells directly preventing peptide release. ETH is expressed in the epitracheal gland located along the major tracheal branches (O'Brien and Taghert, 1998; Park et al., 2002). No expression of GFP or dsRed was seen in these cells but expression was seen in the brain and ventral nerve cord in locations that might correspond to the EH and/or CCAP cells (Figure 2) (Langlais et al., 2004).

To determine whether there was overlap in the expression of EH or CCAP with either *Gyc-89Da* or *Gyc-89Db* we generated larvae that contained *p89Da-GFP* or *p89Db-GFP* with *UAS-dsRED* and the upstream region of EH coupled to GAL4 (McNabb et al., 1997) or the upstream region of CCAP coupled to GAL4 (Park et al., 2003). Figure 5 shows images of the CNS from these larvae. EH is expressed in a single pair of cells in the ventral midline of the brain (Horodyski et al., 1993). Figures 5A and 5B clearly show that there was no overlap in the expression of dsRed driven by the upstream region of EH with the GFP driven by the promoter of either *Gyc-89Da* or *Gyc-89Db*. CCAP is expressed in a row of paired cells along the lateral edges of the ventral nerve cord (Park et al., 2003) and again there was no overlap with the expression of *Gyc-89Da* or *Gyc-89Db* (Figures 5C and 5D).

The row of cells lying adjacent to the CCAP cells appears to be in a similar position to peptidergic neurons that express the transcription factor *dimmed* (Hewes et al., 2003). To determine whether any of the *Gyc-89Da* or *Gyc-89Db* cells also expressed *dimmed*, we generated larvae that contained the *dimmed-GAL4*, *UAS-dsRed* and *p89Da-GFP* or *p89Db-GFP* transgenes. Examination of the nervous tissue of these larvae showed that paired cells in the ventral nerve cord co-expressed *Gyc-89Da* and *dimmed* (Figure 5E). These cells appeared to be in a similar position to the dorsal furin-containing neurons (d1-d11) described in Hewes et al (2003). These furin-expressing cells also express the transcription factor *apterous* and it has been suggested that neuropeptides from these cells are involved in the control of larval ecdysis (Park et al., 2004). We also tested whether there was overlap in the expression patterns of the *Gyc-89Da* or *Gyc-89Db* cells using an *apterous* enhancer trap line (Park et al., 2004). Examination of the larval CNS from animals bearing *UAS-dsRed*, *ap-GAL4* and *p89Da-GFP* or *p89Db-GAL4* showed no overlap in expression of either *Gyc-89Da* and *Gyc-89Db* and *apterous* (data not shown). It therefore appears that the subset of *Gyc-89Da* neurons that are *dimmed* positive are *apterous* negative.

### Reduced levels of cGMP in *Gyc-89Da* and *Gyc-89Db* neurons has no effect on larval ecdysis or eclosion

Several studies have shown that cGMP mediates the action of EH and although the identity of the guanylyl cyclases (GC) that is activated is not known, there is evidence that it is an atypical sGC (see Morton and Simpson, 2002). Because we were specifically targeting neurons that expressed this subclass of sGC, it was possible that cGMP in these neurons was required for, or modulated, ecdysis and eclosion. To test for this we expressed a cGMP-specific phosphodiesterase (bovine PDE5) in the *Gyc-89Da* and *Gyc-89Db* neurons using a *UAS-bPDE5* fly line (Broderick et al., 2004). Progeny of these crosses all developed and eclosed normally with no differences in either the time taken for development or the number of animals that successfully eclosed compared to either of the parental lines (data not shown). We also closely observed the final stages of adult eclosion as described above and could detect no differences in time taken for any of the stages prior to eclosion (data not shown).



### Expression of TNTa in the *Gyc-89Da* neurons is necessary early in adult development to prevent eclosion

The action of TNTa in the *Gyc-89Da* neurons to prevent eclosion could be due to its acute action at the time of eclosion or it could be due to a disruption of an earlier developmental event that is subsequently required for eclosion. To distinguish between these two possibilities we co-expressed GAL80ts, a temperature sensitive inhibitor of GAL4, with *p89Da-GAL4* and *UAS-TNTa* to control the temporal expression of TNTa. The yeast GAL80 protein blocks GAL4 activity by binding to its transcriptional activation domain, a function that is retained when expressed in *Drosophila* (Yocum and Johnston, 1984; Zeidler et al., 2004). A temperature-sensitive version of GAL80 (GAL80ts) was created by inserting a temperature-controlled yeast intein into a crucial region of the GAL80 peptide and placing this construct under the control of a ubiquitous tubulin promoter (*tubP-GAL80ts*) (McGuire et al., 2003). We then generated flies that were homozygous for *p89Da-GAL4* and *tubP-GAL80ts*, which were then crossed with *UAS-TNTa* and *UAS-TNTi* flies. At the permissive temperature, 18°C, GAL80ts inhibits GAL4 action preventing TNT expression while at 30°C GAL80ts is inactive, allowing GAL4 to drive expression of TNT. To test the effectiveness of this system we first crossed *tubP-GAL80ts; p89Da-GAL4* flies to *UAS-dsRed* flies and raised the offspring at either 18°C or 30°C. The larvae raised at 30°C displayed robust dsRed expression whereas larvae raised at 18°C displayed no dsRed expression in the CNS or PNS, but did retain some expression in the salivary glands, indicating that GAL80ts function was mostly inhibited at this temperature (data not shown). To confirm that we could use this system to regulate TNT expression during adult development we shifted animals containing *tubP-GAL80ts; p89Da-GAL4* flies and *UAS-dsRed* transgenes from 18°C to 30°C about mid-way through adult development (96hr at 18°C) and examined expression of dsRed in the brain various times after temperature shift. Examples of these experiments are shown in Figure 6. No expression was seen in the absence of a temperature shift (Figure 6A), low levels of expression were seen 7 hr after the temperature shift (Figure 6B) and robust expression was seen 16hr after the shift to 30°C (Figure 6C).

To determine when TNT needed to be expressed to block eclosion we crossed *tubP-GAL80ts; p89Da-GAL4* flies with *UAS-TNTa* and *UAS-TNTi* flies and raised the progeny at 18°C until the white puparium (WP) stage. At various times after WP, the animals were transferred to 30°C and the number that subsequently eclosed was noted. The results of this experiment are shown in Figure 6D. When animals were shifted to 30°C 24-48hrs after WP the majority failed to eclose if they expressed TNTa whereas control animals expressing TNTi had a high eclosion rate. By contrast, when animals were shifted later in development, no difference was seen in the rates of eclosion between those expressing TNTa and TNTi. This suggests that TNTa expression disrupted a developmental event early in adult development that is required for eclosion.

### Expression of TNTa in the *Gyc-89Db* neurons early in adult development also prevents eclosion

Because TNTa expression in *Gyc-89Db* neurons blocked the first larval ecdysis it was not possible to determine if *Gyc-89Db* neurons were required for all ecdyses. To test this we again used the temperature sensitive GAL80. Flies were generated that were homozygous for both *p89Db-GAL4* and *tubP-GAL80ts* and these were crossed with both *UAS-TNTa* and *UAS-TNTi*. The progeny were maintained at 18°C and then shifted to 30°C various times after WP and the number that successfully eclosed was noted. These data are also shown in Figure 6D. The results were very similar as those found with *Gyc-89Da*. When the temperature shift occurred early in adult development, eclosion was subsequently blocked whereas shifting at later stages had little effect on eclosion rates. Interestingly, the critical time in development was also similar to that found for expression of TNTa in the *Gyc-89Da* neurons. When TNT was expressed earlier than 48 hrs after WP, the majority of the animals failed to eclose.

We also shifted the *p89Db-GAL4; tubP-GAL80ts; UAS-TNTa* and *UAS-TNTi* animals from 18°C to 30°C prior to the WP stage to determine whether they could successfully form puparia. Animals expressing *UAS-TNTa* that were shifted during the first instar failed to form puparia (2.7% success) whereas those shifted during the second and third instar were more successful (70.5% and 93.8% respectively). Soon after puparium formation, *Drosophila* larvae undergo a process known as head eversion, which appears to be the behavior equivalent to pupal ecdysis in other holometabolous insects (Zdárek and Friedman, 1986). All of the larvae containing *p89Db-GAL4; tubP-GAL80ts* and *UAS-TNTa* that were shifted to 30°C during the second and third instar that successfully formed puparia, also successfully completed head eversion (data not shown). Thus neurons that express *Gyc-89Db* are required for larval and adult ecdyses, but not head eversion.

## Discussion

### Expression patterns of *Gyc-89Da* and *Gyc-89Db*

In our previous study we used in situ hybridization to show that the atypical sGCs in *Drosophila* were expressed in a subset of central neurons and peripheral chemosensory neurons (Langlais et al., 2004; Morton et al., 2005a). In the present study we extended these observations by generating fly lines that expressed either GFP or GAL4 under the control of the upstream regions for two of the three atypical sGCs, *Gyc-89Da* and *Gyc-89Db*. We first confirmed that the expression patterns generated by the predicted promoter regions for each of the genes matched the expression patterns revealed by in situ hybridization. For the peripheral sensory neurons, we compared the number of cells labeled and their positions in the embryonic in situ preparations with third instar larvae expressing GFP under the direct control of the predicted promoter regions for each gene. In general, the number and position of the cells labeled with either technique matched well. These included neurons innervating the dorsal and terminal organs, lateral and ventral external sensilla and the terminal sensory cones. These data strongly suggested that the sequences of DNA 5' to *Gyc-89Da* and *Gyc-89Db* that we used in our constructs were sufficient to mimic the expression patterns of these genes.

In the earlier in situ hybridization studies, we used the locations of the cells and cell counts to conclude that in most, if not all, of the peripheral sensory neurons all three of the atypical sGCs were co-expressed in the same cells (Langlais et al., 2004; Morton et al., 2005a). Using the GFP and GAL4 reporter lines we now show more clearly the extent of the overlap between *Gyc-89Da* and *Gyc-89Db* and in contrast to the in situ data, the present study shows relatively little overlap in the expression pattern of these two subunits. There are two primary reasons for the apparent overlap of expression seen using in situ hybridization. Firstly, the sequence similarity between *Gyc-89Da* and *Gyc-89Db* makes it difficult to generate gene-specific probes to each gene possibly resulting in some non-specific labeling. Secondly, most of the cell counts made with the simultaneous application of two probes used a probe to *Gyc-88E* in combination with either *Gyc-89Da* or *Gyc-89Db*. The cell counts were similar in both cases to the cell counts using single probes (Langlais et al 2004) suggesting that all three genes were co-expressed in the same cells. If we had carried out cell counts combining short, gene-specific probes to both *Gyc-89Da* and *Gyc-89Db*, we might have been able to see more cells than with either probe alone, which would have suggested a lack of co-expression. The results from the present study using expression of fluorescent proteins showed that in the olfactory and gustatory neurons there is no overlap in the expression patterns, with each subunit expressed in about 4 neurons in each ganglion. Along the lateral body wall each of the single neurons innervating external sensilla co-expressed *Gyc-89Da* and *Gyc-89Db* whereas the tracheal dendritic neurons only expressed *Gyc-89Db*. The terminal sensory cones showed a similar pattern seen with the in situ hybridization results – a single neuron in each cone expressed both atypical sGCs. In the CNS it had not been previously possible to examine co-expression patterns because of the

number cells and lack of morphological markers. The reporter constructs show clearly that *Gyc-89Da* and *Gyc-89Db* are not co-expressed in any CNS neurons. The only locations where they are co-localized are in some of the intrinsic cells of the corpora cardiaca. Because *Gyc-89Da* and *Gyc-89Db* are only enzymatically active when co-expressed with the third atypical sGC, *Gyc-88E* (Langlais et al., 2004; Morton et al., 2005a), we assume that *Gyc-88E* is also expressed in all the central and peripheral cells that express *Gyc-89Da* and *Gyc-89Db*. The in situ hybridization experiments support this, but as we have not been able to define the promoter region for *Gyc-88E* or generate antisera against *Gyc-88E*, we have not been able to show this directly.

The identity of the *Gyc-89Da* and *Gyc-89Db* cells in the CNS are unknown at this time. We tried a variety of markers that appeared to have similar expression patterns but were unable to see any overlap. These include cells that contain the neuropeptides EH and CCAP and the transcription factor *apterous*. The transcription factor *dimmed* is expressed in a wide variety of peptidergic neurons (Hewes et al., 2003) and showed co-expression with a small subset of *Gyc-89Da* neurons but had no overlap in the CNS with *Gyc-89Db*. This suggests that this subset of *Gyc-89Da* neurons is peptidergic, but the identity of the peptide is unknown.

### Functions of the *Gyc-89Da* and *Gyc-89Db* neurons

We used the neurotoxin, TNT, to determine the function of the neurons that express *Gyc-89Da* and *Gyc-89Db*. TNT blocks synaptic transmission by cleaving the synaptic vesicle protein, synaptobrevin (Schiavo et al., 1992; Link et al., 1992) and expression of TNT in specific neurons in *Drosophila* has helped define neuronal networks involved in a variety of behaviors (Martin et al., 2002). When we expressed TNT in either the *Gyc-89Da* or *Gyc-89Db* neurons we found that the animals died at three specific stages (Figure 3). TNT expression in *Gyc-89Da* neurons caused about half of the animals to die at the late larval stage and the remainder of the animals to die just prior to the onset of the eclosion motor program. The lethality of third instar larvae was associated with burrowing into the food and could be rescued by allowing the animals to develop in a thin layer of food. We have shown that the activity of the atypical sGCs are regulated by oxygen and have suggested that they could act as oxygen sensors (Morton, 2004b). If the *Gyc-89Da* neurons are excited by reduced oxygen levels and activate hypoxia-associated behaviors, when synaptic transmission of these neurons is blocked by TNT the animals would no longer respond to hypoxia. One response of larvae to hypoxia is to withdraw from the food and activate searching behaviors (Wingrove and O'Farrell, 1999). Our results are consistent with the *Gyc-89Da* neurons either detecting hypoxia or in activating the hypoxia escape behavior. If synaptic transmission is blocked in these neurons the animals would fail to respond to hypoxia, continue to burrow into the food and suffocate. Most of the larval lethality that we observed was late in the third instar, presumably because at this time locomotion increases as the larvae crawl out of their food to seek a location to pupate. Wingrove and O'Farrell (1999) have shown that the hypoxia escape response is likely mediated by a cGMP/PKG pathway and we have preliminary data that show that cGMP in the *Gyc-89Da* neurons is necessary for a normal hypoxia escape response (Morton et al., 2005b).

The other major period of lethality for animals when TNT was expressed in the *Gyc-89Da* neurons was just prior to adult eclosion. All of the stages that precede the onset of the eclosion motor program occurred normally and in the appropriate order until the final stage – head expansion. This time corresponds closely with the time at which defects in eclosion were first detected in flies lacking the neurons that contain EH (McNabb et al., 1997), a neuropeptide known to trigger ecdysis and eclosion in most, if not all, insects (Truman et al., 1981). The simplest interpretation of our findings is that *Gyc-89Da* is expressed in EH neurons and expression of TNT in these cells prevented EH release. This is not the case as there was no overlap in EH and *Gyc-89Da* expression patterns. Cells that are immediately upstream and

downstream of EH in the pathway that regulates eclosion are the Inka cells of the epitracheal glands and the CCAP neurons (see Ewer and Reynolds, 2002; Clark et al., 2004). No overlap in the expression pattern of *Gyc-89Da* with either of these cells was seen. It is possible that the *Gyc-89Da* neurons act further upstream of the EH cells, although the *Gyc-89Da* neurons must affect a larger number of neurons associated with eclosion than the EH cells as the phenotype of TNT expression in the *Gyc-89Da* neurons is much more severe than the EH cell deletion. Almost all the *p89Da-TNTa* animals failed to eclose whereas about a third of the flies lacking EH cells complete development and eclose (McNabb et al., 1997). As with the EH cell deletions, expression of TNT in the *Gyc-89Da* neurons prevented normal tanning and wing expansion.

Expression of TNT in the *Gyc-89Db* neurons also phenocopied the effects of eliminating a neuropeptide associated with eclosion. ETH acts both upstream and downstream of EH as part of a positive feedback loop (Clark et al., 2004) and mutations that eliminate the ETH gene cause lethality at the first larval ecdysis (Park et al., 2002). The timing of death in ETH  $-/-$  larvae (Park et al., 2002) is very similar to larvae expressing TNT in the *Gyc-89Db* neurons – just prior to air filling of the tracheae. In wild type larvae it only takes about 2 min between tracheal collapse and air filling of the tracheae (Park et al., 2002; Clark et al., 2004). Interestingly, in the EH cell deletions, many of the larvae also failed to air fill their tracheae and this was a major cause of lethality (McNabb et al., 1997). Thus elimination of either ETH or EH results in a similar phenotype to blocking neurotransmission in the *Gyc-89Db* neurons. As described above for the *Gyc-89Da* neurons a simple explanation for this is that TNT is expressed in ETH, EH or CCAP cells preventing the release of the peptides. This is not the case as we see no overlap in expression patterns with any of these peptides and *Gyc-89Db*. Additional peptides believed to be involved with activation of the ecdysis behavior program include peptides in the *apterous* cells (Park et al., 2004), corazonin (Kim et al., 2004) and the products of the *hugin* gene (Meng et al., 2002). Although we saw no overlap with *apterous* it is possible that the release of other peptides is prevented by expression in TNT in the *Gyc-89Da* or *Gyc-89Db* neurons. Although TNT blocks synaptic transmission at conventional synapses in *Drosophila*, there is little experimental support that in *Drosophila* TNT will block peptide release (Martin et al., 2002).

Another possibility is that synaptic transmission in *Gyc-89Db* neurons is directly required for successful air filling of tracheae. The physiological basis for air filling the tracheae at each molt is not known although the ETH neuropeptide is involved (Park et al., 2002). *Gyc-89Db* is expressed (in the absence of *Gyc-89Da*) in sensory neurons that are closely associated with tracheae (Figure 2B). If these neurons signal to the CNS when apolysis has progressed sufficiently to trigger air filling, failure of synaptic transmission in these cells could account for the inability of the animal to successfully fill its tracheae with air.

### Effects of temporal control of TNT expression

The data discussed above supported the model that synaptic transmission in neurons that expressed *Gyc-89Da* and *Gyc-89Db* was required for either the activation or the generation of the eclosion and ecdysis motor programs. This model predicted that TNTa expression in these neurons just prior to ecdysis or eclosion is sufficient to block the behavior. Our experiments using GAL80ts to control the temporal expression of TNTa showed that this was not the case. TNTa expression about 100 hr before eclosion is required to block eclosion. This delay is not explained by the time it takes for the inhibition of GAL4 activation to be removed. McGuire et al., (2003) found that 30 min after the temperature shift a reporter gene was detectable and half maximal expression was seen after 3 hr. Our data showed that expression of the red fluorescent protein, dsRed, was detectable 7 hr after the temperature shift and was robust 16 hrs later.

Our findings suggest that TNT expression probably blocks a developmental event that is required for the subsequent expression of eclosion behaviors. Previous studies have also shown developmental effects of TNT expression in *Drosophila* neurons. In the embryonic neuromuscular system, expression of TNT in motor neurons resulted in the expected blockage of neuromuscular synaptic transmission and also reduced synaptic input to the motor neurons (Baines et al., 1999). In a detailed analysis of the effects of TNT expression in the developing optic lobe, Hiesinger et al. (1999) found that cell survival, axonal pathfinding and target recognition were unaffected by TNT expression, but morphological alterations in photoreceptor terminals was observed between 25 and 50% of adult development. This time corresponds to the window of sensitivity that we observed for the blockage of adult eclosion. This suggests that expression of TNT in the *Gyc-89Da* and *Gyc-89Db* neurons disrupts their connectivity and leads to a blockade in eclosion. It is interesting that the *Gyc-89Da* neurons are not required for larval ecdysis, whereas the *Gyc-89Db* neurons are, but for adult eclosion TNT expression in either population of cells blocked the behavior. In the larval CNS there was very little overlap of expression, and similarly in the adult CNS few, if any, neurons co-express both *Gyc-89Da* and *Gyc-89Db*.

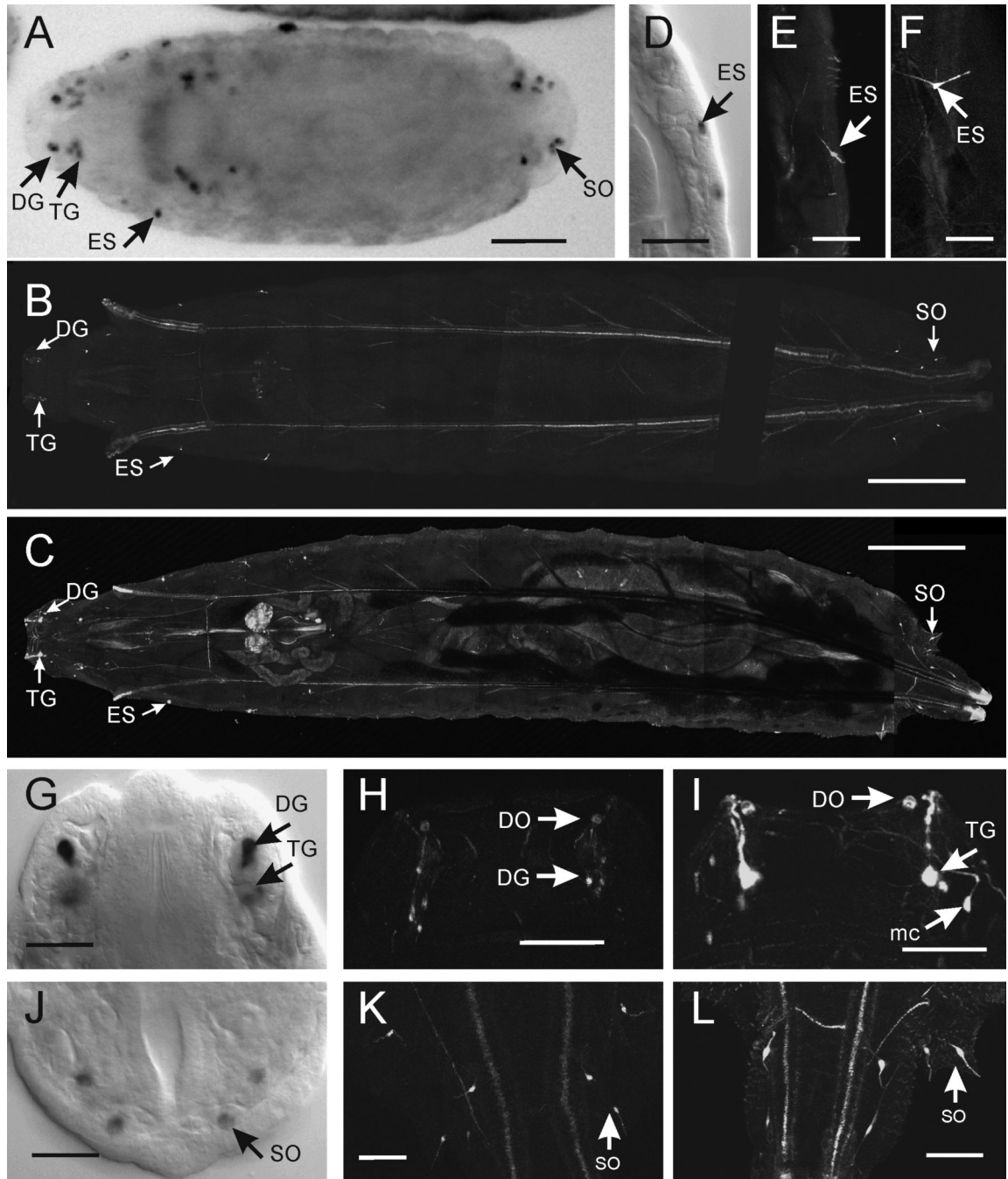
In summary, by using targeted expression of TNT in neurons that express two atypical soluble GCs, we show that synaptic transmission in neurons that express *Gyc-89Da* are required for adult eclosion and neurons that express *Gyc-89Db* are required for larval ecdysis and adult eclosion. By contrast, neither of these populations of neurons is required for head eversion. The use of the driver lines also enabled us to more completely map the expression patterns of these two GC subunits and we found some co-expression in peripheral sensory neurons, but almost no overlap in CNS expression.

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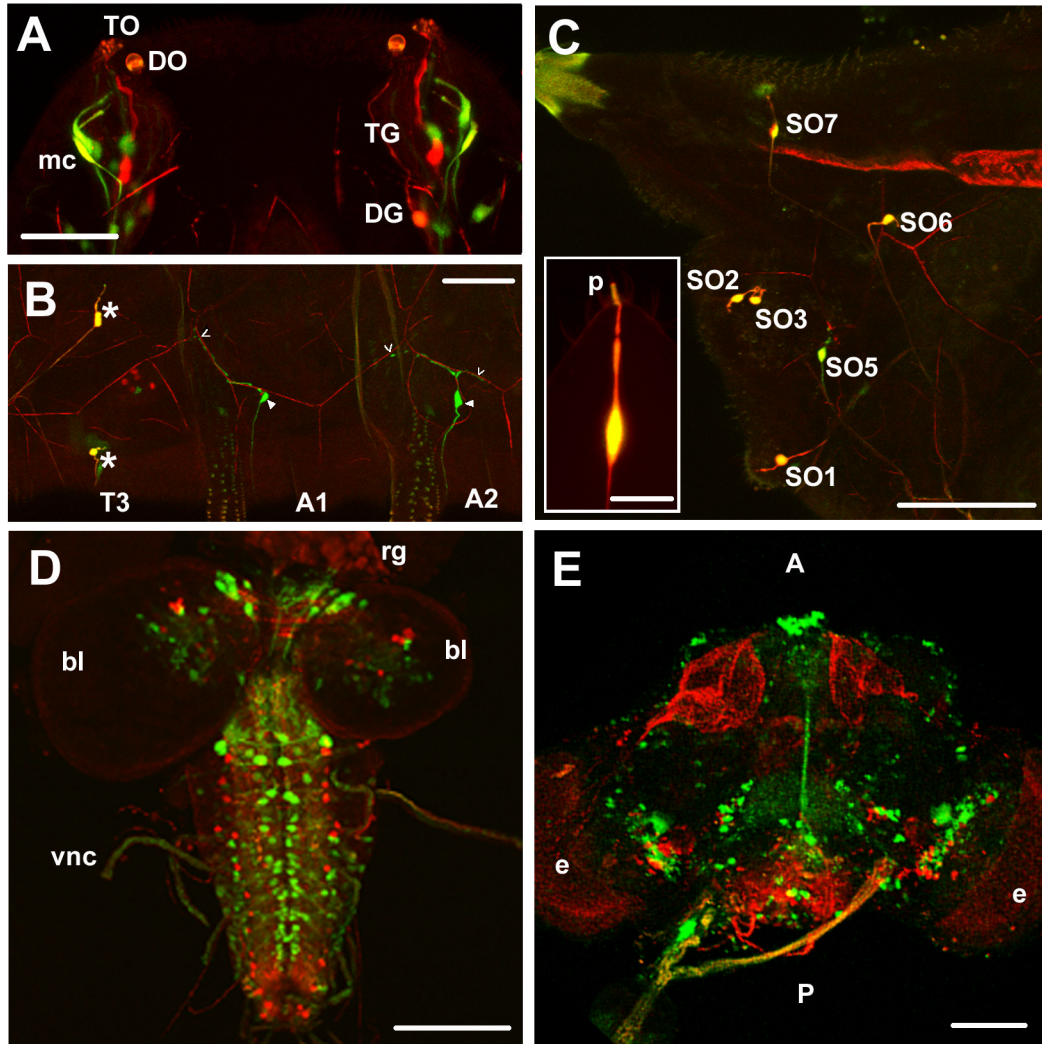


**Figure 1.**

Upstream regions of *Gyc-89Da* and *Gyc-89Db* drive GFP expression in sensory neurons in larvae in a similar pattern as seen for in situ hybridization for *Gyc-89Da* and *Gyc-89Db* in embryos. Second instar larvae containing the *p89Da-GFP* (B, E, H & K) or *p89Db-GFP* (C, F, I & L) transgenes were examined for GFP fluorescence and compared with embryos stained with a digoxigenin-labeled riboprobe for *Gyc-89Db* (A, D, G & J). Anterior is to the left (A-C) or up (D-L). A-C. Low magnification showing the three populations of sensory neurons stained: chemosensory neurons in the dorsal ganglion (DG) and terminal ganglion (TG), external sensilla (ES) neurons and neurons in the terminal sensory organs (SO). D-F. High magnification of the lateral body wall showing a neuron innervating the external sensilla (ES).



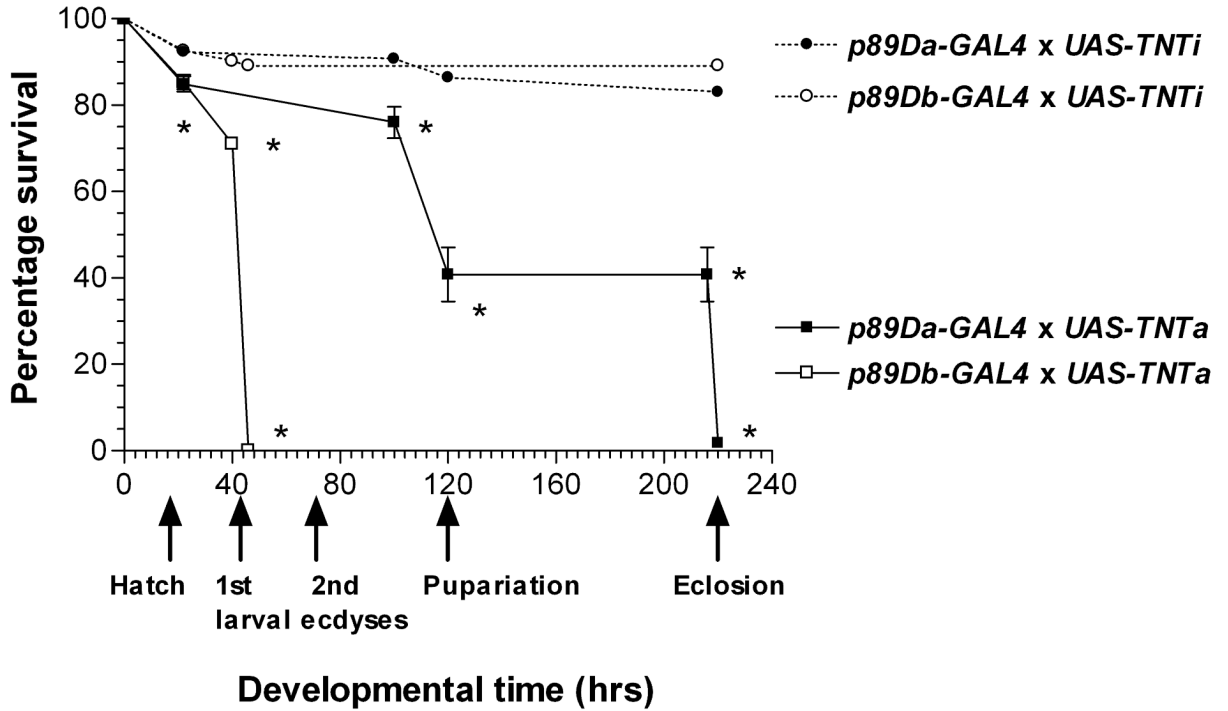
**G-I.** High magnification of the anterior end of embryo or larvae showing neurons in the dorsal ganglion (DG) and terminal ganglion (TG) that innervate the dorsal organ (DO) and terminal organ respectively. *p89Db-GFP* also drives GFP expression in neurons in the monoscolopodial organ (mc). **J-L.** High magnification of the posterior end of embryo or larvae showing a single neuron innervating each terminal sensory organ (SO). Fluorescence seen in tracheae is due to autofluorescence. Scale bars represent 500 $\mu$ m (**B & C**), 200 $\mu$ m (**A**), 100 $\mu$ m (**E,F,H,I,K & L**) and 50 $\mu$ m (**D,G & J**).



**Figure 2.**

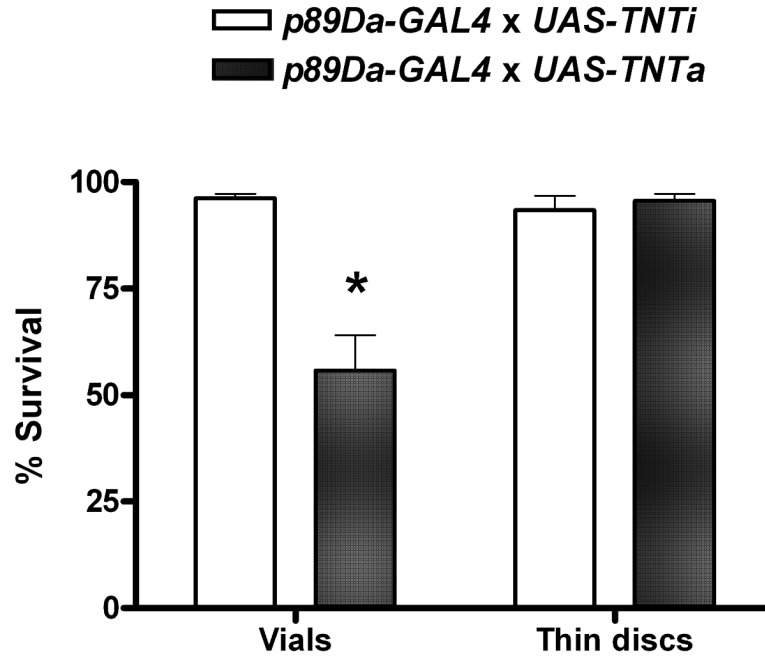
Relatively few neurons co-express *Gyc-89Da* and *Gyc-89Db*. Flies were crossed to generate animals containing *p89Da-GFP*, *p89Db-GAL4* and *UAS-dsRED*. Each panel shows a confocal z-stack, collapsed into a single plane with the red color representing cells expressing *Gyc-89Da* and the green color representing cells expressing *Gyc-89Db*. **A-D** third instar larvae, **E** adult. **(A)** Dorsal view (anterior is up) of the terminal organ (TO), terminal ganglion (TG), dorsal organ (DO) and dorsal ganglion (DG). Both ganglia contain cells that express either *Gyc-89Da* or *Gyc-89Db* but no cell shows co-expression of both subunits. Also shown are two neurons that are part of the monoscolopidial organ (mc) that only express *Gyc-89Db*. **(B)** Lateral body wall showing the third thoracic (T3) and first two abdominal segments (A1 & A2). In T3 two neurons express both *Gyc-89Da* and *Gyc-89Db* (\*): a single *les* neuron (upper) and a single *ves* neuron (lower). In A1 and A2 a single *td* neuron expresses only *Gyc-89Db* (solid arrow heads). The fine red lines are trachea closely associated with fine branches from the two *td* neurons (open arrow heads) **(C)** Terminal segment showing the caudal sensory cones (SO1-SO7). Each cone contains a single neuron that co-expresses both *Gyc-89Da* and *Gyc-89Db*. The inset shows a single neuron extending a neurite to a cuticular peg sensillum (p). **(D)** Larval CNS showing extensive expression of each subunit but no overlap in brain lobes (bl) and ventral nerve cord (vnc). Expression is also seen in the ring gland (rg). **(E)** Adult brain

also shows extensive, non-overlapping expression of *Gyc-89da* and *Gyc-89Db*. Fluorescence seen in tracheae is due to autofluorescence. Scale bar = 40 $\mu$ m (A), 50 $\mu$ m (C), 100 $\mu$ m (B,D,E), 25 $\mu$ m (C, insert).



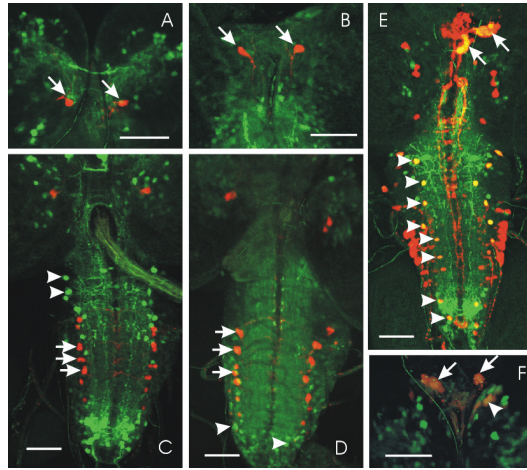
**Figure 3.**

Effect of expression of TNT in the *Gyc-89Da* and *Gyc-89Db* on survival during development. Flies expressing GAL4 under the control of the *Gyc-89Da* and *Gyc-89Db* promoters (*p89Da-GAL4* and *p89Db-GAL4* respectively) were crossed with flies expressing the active (TNTa) and inactive (TNTi) forms of the light chain of tetanus toxin (TNT) under the control of the UAS promoter (*UAS-TNTa* and *UAS-TNTi* respectively) and the survival of the progeny determined at the stages shown. Survival rates for progeny of *UAS-TNTa* flies are shown with a solid line and progeny from *UAS-TNTi* flies with a broken line and those from *p89Da-GAL4* flies with solid symbols and from *p89Db-GAL4* flies with open symbols. The data represent the percent survival calculated from the number of eggs laid and represent the mean  $\pm$  SEM of at least 3 trials using up to 300 progeny per trial. For each developmental stage the experimental and control groups were compared statistically using paired t-tests and asterisk indicates  $P < 0.05$ .

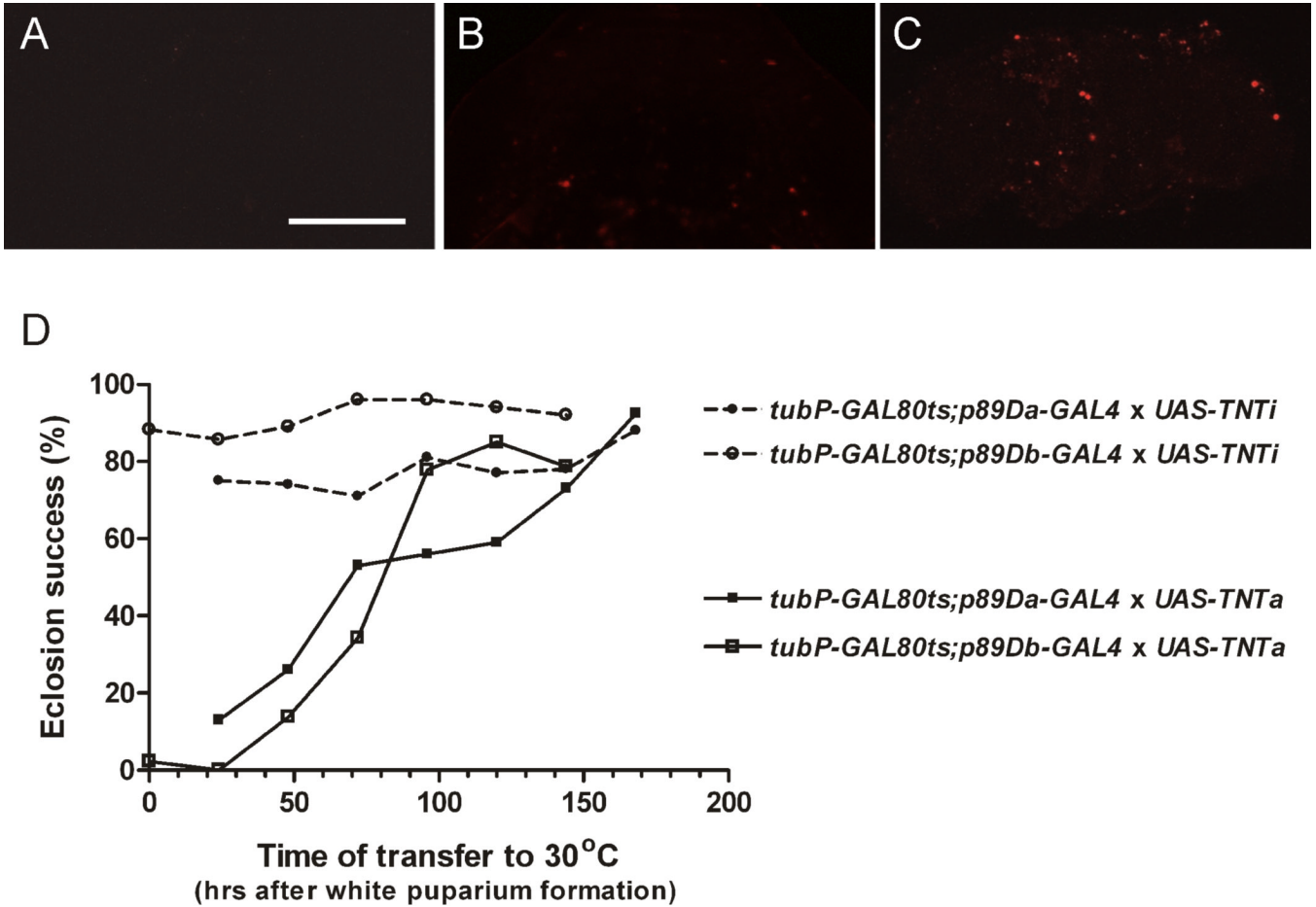


**Figure 4.**

Rescue of lethality at the third larval instar of *p89Da-GAL4 × UAS-TNTa* progeny by preventing burrowing. Early third instar larvae were either placed in vials or on a thin disc of food and the number that successfully formed puparia were counted. The data represents the percent survival (mean ± SEM) of 4 trials each using 100-300 animals. The asterisk represents a significant (students t test,  $p < 0.05$ ) reduction in survival between progeny from *UAS-TNTa* flies (solid bars) compared to *UAS-TNTi* (open bars) when raised in vials whereas no significant difference in survival was seen when larvae were raised on thin food discs. The majority of the dead animals found in the vials were late third instar larvae found deep in the food.



**Figure 5.** *Gyc-89Da* and *Gyc-89Db* are not expressed in EH or CCAP cells, but are expressed in some peptidergic cells. Crosses were made to generate larvae containing the following transgenes **A.** *p89Da-GFP*, *EH-GAL4* and *UAS-dsRed*, **B.** *p89Db-GFP*, *EH-GAL4* and *UAS-dsRed*, **C.** *p89Da-GFP*, *CCAP-GAL4* and *UAS-dsRed*, **D.** *p89Db-GFP*, *CCAP-GAL4* and *UAS-dsRed*, **E.** *p89Da-GFP*, *dimmed-GAL4* and *UAS-dsRed*, **F.** *p89Db-GFP*, *dimmed-GAL4* and *UAS-dsRed*. In **A** and **B** the EH neurons are indicated with arrows and in **C** and **D** the arrows indicate CCAP neurons. In **E** and **F** the arrowheads indicate the neurons in the CNS that express both *dimmed* and *Gyc-89Da* or *Gyc-89Db*. Additional cells that express the atypical sGCs and *dimmed* are intrinsic cells in the ring gland, which are indicated with arrows. The scale bar represents 50 $\mu$ m.



**Figure 6.**

TNT expression early in adult development is required in both *Gyc-89Da* and *Gyc-89Db* neurons to block eclosion. **A**, **B** and **C**. Expression of dsRed in CNS of *p89Da-GAL4; tubP-GAL80ts; UAS-dsRed* developing adults. Animals were raised at 18°C until 96 hr after white puparium formation and then transferred to 30°C. After 0 (**A**), 7 (**B**) and 16 (**C**) hours the brain was removed and observed under fluorescence optics. Expression of dsRed in the CNS was seen 7 hrs after transfer to 30°C. **D**. Eclosion rates of flies that express TNTa at different times of adult development in the *Gyc-89Da* (solid symbols) and *Gyc-89Db* (open symbols) neurons. Larvae containing *tubP-GAL80ts* and *p89Da-GAL4* or *p89Db-GAL4* and *UAS-TNTa* (solid line) or *UAS-TNTi* (broken line) were raised at 18°C until white puparium formation and then transferred to 30°C at the times shown. The majority of animals transferred early in adult development (24-48hrs after white puparium) had a low eclosion rate whereas those transferred after about 100hrs showed little difference in their eclosion rates compared to controls.

Table 1

Sensory neuron group	Gvc-89Da		Gvc-89Db		p89Dd-GFP	Embryo in situ	p89Da-GAL4; UAS-dsRed	p89Db-GAL4; UAS-dsRed	p89Dd-GFP p89Db- GAL4; UAS-dsRed
	Embryo in situ	p89Da-GFP	p89Db-GFP	p89Dd-GFP					
Dorsal ganglion	3-5	4-5	4	3	4	4-5	4	4	0
Terminal ganglion	2-3	2-3	3-4	2-3	3-4	2-5	3-4	3-4	0
Thoracic sensilla	2	2	2	2	2	2	2	2	2
Abdominal sensilla	0	0	0	1	1	1	1	1	0
Terminal sensory cone sensilla	6	6-7	7	7	7	6	(A1-2)	(A1-3)	7

Summary of cell counts from embryo in situ hybridization preparations and direct observations of third instar larvae from promoter-GFP and promoter-GAL4 × UAS-dsRed lines. The embryo in situ hybridization data was compiled from previous studies (Langlais et al., 2004; Morton et al., 2005a). For the GFP and dsRed expression data, at least 5 animals were observed from each of the transformed lines.