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Synaptogenesis in the Giant-Fibre System of *Drosophila*: interaction of the Giant Fibre and its major motorneuronal target

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SUMMARY

The tergotrochanteral (jump) motorneuron is a major synaptic target of the Giant Fibre in *Drosophila*. These two neurons are major components of the fly's Giant-Fibre escape system. Our previous work has described the development of the Giant Fibre in early metamorphosis and the involvement of the *shaking-B* locus in the formation of its electrical synapses. In the present study, we have investigated the development of the tergotrochanteral motorneuron and its electrical synapses by transforming *Drosophila* with a *Gal4* fusion construct containing sequences largely upstream of, but including, the *shaking-B(lethal)* promoter. This construct drives reporter gene expression in the tergotrochanteral motorneuron and some other neurons. Expression of green fluorescent protein in the motorneuron allows visualization of its cell body and its subsequent intracellular staining with Lucifer Yellow. These preparations provide high-resolution data on motorneuron morphogenesis during the first half of pupal development. Dye-coupling reveals onset of gap-junction formation between the tergotrochanteral motorneuron and other neurons of the Giant-Fibre System. The medial dendrite of the tergotrochanteral motorneuron becomes dye-coupled to the peripheral synapsing interneurons between 28 and 32 hours after puparium formation. Dye-coupling between tergotrochanteral motorneuron and Giant Fibre is first seen at 42 hours after puparium formation. All dye coupling is abolished in a *shaking-B(neural)* mutant. To investigate any interactions between the Giant Fibre and the tergotroachanteral motorneuron, we arrested the growth of the motorneuron's medial neurite by targeted expression of a constitutively active form of Dcdc42. This results in the Giant Fibre remaining stranded at the midline, unable to make its characteristic bend. We conclude that Giant Fibre morphogenesis normally relies on fasciculation with its major motorneuronal target.

Key words: Dye coupling, Gap junction, Innexin, *shaking-B*, TTMn, *Drosophila melanogaster*

INTRODUCTION

The Giant Fibre System (GFS) is the most extensively studied network of single-identified neurons in *Drosophila* (see, for example, Blagburn et al., 1999; Thomas and Wyman, 1983). Uniquely, it is the only neural circuit in the *Drosophila* CNS which is currently amenable to both genetic and physiological approaches. The Giant Fibres (GFs) are a pair of large brain interneurons that are activated by visual and wind stimuli and mediate the rapid escape behaviour of flies (Thomas and Wyman, 1983). Each axon descends into the mesothoracic neuromere of the thoracic ganglion, where it synapses directly with the tergotrochanteral (jump) muscle motorneuron (TTMn). In addition, it synapses indirectly, via the peripherally synapsing interneuron (PSI), with motorneurons that activate the dorsal longitudinal flight muscles (DLMns; King and Wyman, 1980; Tanouye and Wyman, 1980). Recent EM studies have shown that the GF makes mixed electrical and chemical synapses with both the TTMn and the PSI (Blagburn et al., 1999).

A focus of our work is to investigate the mechanisms involved in the formation of this simple neuronal circuit. In a previous study, we used the A307 enhancer trap line in which Gal4 is expressed in a number of neurons, notably the GF, to examine GF development. This work showed that the GF grows into the thoracic ganglion during early pupation and, by about 30% of pupal development, reaches a position where the synapse with the TTMn could be formed (Phelan et al., 1996). However intracellular filling with Lucifer Yellow of the GF axon was not possible before 45 hours APF (after puparium formation) because of its small axon diameter. By this time the GF is already dye-coupled to the TTMn, indicating the presence of electrical synapses. To obtain a more complete understanding of the development of this, and other electrical synapses in the GFS, we needed to investigate the development of TTMn. The A307 line expresses very weakly in TTMn (Allen et al., 1998), making it difficult to describe precisely TTMn's morphogenesis and its first contact with the GF.

One strategy would be to make a reporter construct in which strong expression in TTMn is not accompanied by expression

in GF. The *shakB* locus, some alleles of which cause perturbed escape behaviour (Thomas and Wyman, 1984), provided a promising avenue. There are several transcripts produced from this locus (Zhang et al., 1999); flies carrying the *shakB*^{2} mutation, which disrupts all ShakB(neural) proteins, have perturbed electrical transmission (Baird et al., 1990; Thomas and Wyman, 1984) and dye coupling (Phelan et al., 1996; Sun and Wyman, 1996) in the GFS. Subsequent work has shown that products of the *shakB* locus are structural components of gap junctions (Phelan et al., 1998a,b). Of particular bearing on the present study, in situ hybridization data suggest that one transcript of this locus, *shakB(lethal)*, may be expressed in several neurons of the GFS, but not the GF itself (Crompton et al., 1995). We therefore made a construct, in which an upstream regulatory region of *shakB(lethal)* was fused to *Gal4*, and used this to drive reporter gene expression in transformed flies.

This strategy proved successful; we found that several neurons of the GFS, including the TTMn, but not the GF, express the construct. Using a *tau-GFP* reporter, we were able to identify the TTMn from very early pupal stages and to impale and stain it with Lucifer Yellow filled microelectrodes. Such stainings provide very detailed information about dendritic development of the TTMn during the pupal stage and make it possible to determine the onset of gap-junction formation between TTMn and other neurons of the GFS. The TTMn establishes its adult morphology during the first 50 hours APF. Dye coupling between the medial dendrite of the TTMn and the PSIs starts between 28 and 32 hours APF whereas dye coupling between TTMn and GF is first seen at 42 hours APF. All dye coupling is abolished in a *shakB(neural)* mutant. This construct also allowed us to target expression of Dcdc42(V12), a constitutively active mutant form of a member of the Rho family of small GTPases, in the TTMn; this arrests growth of TTMn's medial dendrite and renders the GF incapable of forming its characteristic bend. We conclude that fasciculation between the GF and the (already bent) TTMn is a major influence on GF's thoracic morphology.

MATERIALS AND METHODS

Fly stocks and pupal staging

The wild-type strain used in this study was Oregon-R. *shakB*^{2} is a mutation in the *shakB* locus, originally isolated in a screen for behavioural mutants (Homyk et al., 1980). All *Drosophila* stocks were raised at 25°C under standard conditions. For embryo collections, flies were maintained on agar plates at 25°C and allowed to lay eggs overnight. *shakB(lethal)-Gal4* flies constructed for this study (see below) were crossed with flies carrying a *UAS-tau-lacZ* or a *UAS-tau-GFP* fusion construct on the second chromosome (Brand, 1995; Hidalgo et al., 1995). A construct containing a constitutively active form of *Drosophila cdc42*, *UAS-Dcdc42(V12)* (Luo et al., 1994), on the third chromosome, was used to block dendritic outgrowth. Times through metamorphosis are defined as hours after pupal formation (APF). To stage pupae, cultures containing third-instar larvae were checked at intervals of approximately 1 hour and white prepupae (time 0) were either transferred to fresh vials or marked and then left to develop at 25°C. At 25°C, pupation takes 93-106 hours (Bainbridge and Bownes, 1981).

Cloning and P-element mediated transformation

To prepare *shakB(lethal)-Gal4* for transformation, a 4.1 kb *Hin*dIII-*Eco*RI fragment of the genomic clone λ94C2 (J.A.D. unpublished; Fig. 1A), upstream of *shakB(lethal)* and containing its promotor region, was subcloned into *Hin*dIII-*Eco*RI digested pBluescript KS$^+$ vector creating *pP-shakB*. To construct the plasmid for injection, the *Hin*dIII-*Eco*RI fragment of *pP-shakB* was re-isolated, made blunt-ended and cloned into the *Bam*HI polylinker site of *pGaTB* (Brand and Perrimon, 1993). Clones with the promoter fragment in the correct orientation were recovered and named *pPG*. The 7kb *Kpn*I-*Not*I fragment of *pPG* containing the *shakB(lethal)* promoter fragment fused to the *Gal4* coding region was then subcloned into *Kpn*I-*Not*I digested pP{W8} transformation vector, generating *shakB(lethal)-Gal4*. The *shakB(lethal)-Gal4* construct and the P-element helper pπ25.7wc (Karess and Rubin, 1984) were purified by two rounds of CsCl centrifugation and co-injected into embryos of genotype *y w* at concentrations of 400 and 100 μg/ml respectively (Spradling and Rubin, 1982) and the chromosomal locations of the transgene in 15 transformed lines were mapped. For initial characterisation of expression, all 15 lines were crossed to lines carrying *UAS-lacZ*, and embryonic staining examined. The pupal and adult nervous system of three of the strongest staining lines revealed a broadly consistent staining pattern. Results presented in this study were obtained using one of these lines, 2GL152, which mapped to the second chromosome.

β-galactosidase detection

Nervous systems were dissected in *Drosophila* saline (128 mM NaCl, 2 mM KCl, 4 mM $MgCl_2$, 1.8 mM $CaCl_2$, 36 mM sucrose, 5 mM Hepes, pH 7.2) and immediately fixed in 0.5% glutaraldehyde in phosphate-buffered saline (PBS; Oxoid Ltd., Basingstoke, UK) for 15-30 minutes at room temperature. Preparations were then washed in PBT (0.1% Triton-X 100 in PBS) and stored for up to 1 day at 4°C. For X-Gal staining, tissues were equilibrated with staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 3 mM $MgCl_2$ in PBT) for 10 minutes at 37°C and then incubated in freshly prepared 0.08% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Boehringer Mannheim, Lewes, UK) in staining solution at 37°C for 30 minutes to several hours. Preparations were washed several times in PBT, cleared in a 50-100% glycerol series, and mounted on slides in 100% glycerol. For X-gal staining of whole-mount flies, mouth parts, legs, wings, the last four abdominal segments and the gut were removed. Fixation and wash times were doubled. Flies were dehydrated in a 30-100% ethanol series and cleared and mounted in methylsalicylate.

Backfills of muscles

One- to 7-day-old flies carrying the *shakB(lethal)-Gal4* and *UAS-tau-lacZ* transgenes were used to retrogradely label the motorneurons that innervate the DLMns and TTMn. A saturated solution of horseradish peroxidase (HRP, type IV; Sigma, Poole, UK) was prepared in distilled water. The tip of a tungsten needle was immersed in the HRP solution, withdrawn, and allowed to dry. Flies were fixed on double sided tape with either their dorsal or lateral side up. Attachment sites of the muscles were identified using a dissecting microscope and a second sharp tungsten needle was used to make a hole in the cuticle at the attachment sites of the DLMns or the TTMn (Baird et al., 1993) to allow the HRP-coated needle to penetrate the muscle. Flies were transferred to a moist chamber and left for 4-6 hours. After this incubation period, the CNS was dissected out in saline, fixed in 0.5% glutaraldehyde in PBS for 15-30 minutes and washed several times in PBT. Colour reaction was monitored visually by placing the tissue in a diaminobenzidine/H_2O_2 staining solution (Vector Laboratories, Peterborough, UK). The reaction was stopped by several washes in PBT. X-Gal staining was then performed as described above.

Dye fills of single neurons

Nervous systems of pupae and adults carrying *shakB(lethal)-Gal4* and a *UAS-tau-GFP* reporter (Brand, 1995) and, in some instances *UAS-Dcdc42(V12)*, were dissected and mounted immediately onto poly-L-lysine (0.01%; Sigma, Poole, UK) coated glass coverslips in a

chamber containing saline. The TTMn cell body was identified by its GFP expression using a Zeiss Axiophot microscope equipped with fluorescence optics and a 40× water immersion objective. The cell body was impaled, under visual control (Fig. 1B,C), with a borosilicate glass microelectrode (resistance 20-100 mΩ), tip-filled with a solution of 4% Lucifer Yellow (Molecular Probes, Eugene, OR, USA) and backfilled with 2 M $LiCl_2$. After successful penetration of the TTMn cell body, fluorescence was switched off and Lucifer Yellow was injected into the neuron with hyperpolarizing current for up to 30 minutes. The GF axon in the cervical connective was visualised under Nomarski optics and stained with Lucifer Yellow from an intracellular microelectrode as above.

Analysis

Whole-mount preparations with HRP backfills and/or X-Gal staining were photographed on a Zeiss Axiophot microscope equipped with Nomarski optics. Confocal images of Lucifer-Yellow filled neurons were prepared using an argon-krypton-laser scanning confocal microscope (MRC 600, Bio-Rad, Hemel Hempstead, UK). Each picture is the projection of a series of images collected at 1.5-3 μm steps. Confocal images and scanned photographs were prepared using Adobe Photoshop 5 and assembled using Canvas 6.0.

RESULTS

shakB(lethal)-Gal4 is expressed in a subset of postembryonic central neurons

In flies carrying *shakB(lethal)-Gal4* and *UAS-tau-lacZ*, the reporter gene is expressed in relatively few neurons of the CNS. Importantly we see staining in the TTMn. Moreover, as expected from our previous in situ hybridization data, expression is not seen in the GF (Crompton et al., 1995; Fig. 2). In addition, staining of a number of neurons in the brain and thoracic ganglion also reflects *shakB(lethal)* expression (Crompton et al., 1995).

Brain expression

shakB(lethal)-Gal4 flies report *tau-lacZ* expression in optic lobe neurons from about 25 hours APF onwards. Two distinct types of neurons within the medulla can be distinguished (Fig. 2A,B): one set of columnar neurons has cell bodies spaced throughout the medulla with neurites projecting centripetally (Fig. 2B, arrowheads). The other group of neurons has its cell bodies clustered in posterior optic-lobe cortex and projects processes tangentially into the medulla (Fig. 2B, arrows). We could not achieve sufficiently high resolution of the morphology of these optic neurons to classify them according to the nomenclature of Fischbach and Dittrich (1989). Staining of both types of neuron becomes even weaker and fewer cells are stained after about 90 hours APF (data not shown); in adults, expression in these visual neurons could only be observed in two out of six preparations.

In the midbrain, only one pair of neurons expresses *tau-lacZ* (Fig. 2A, arrows). Expression in these neurons begins at about 16 hours APF, by which time each neuron has grown a small posterior process. By 25 hours APF, the processes have continued growing laterally and they have reached the lateral edge of the protocerebum by 40 hours APF. By this time, medial processes have grown across the midline where they appear to contact each other. The medial branches of these protocerebral neurons can be seen in the 52 hours APF preparation (Fig. 2A, arrowheads).

In the suboesophageal ganglion, two pairs of neurons express *tau-lacZ* (Fig. 2A,C). Each neuron projects to the midline, where it arborizes and possibly fasciculates with its contralateral homologue. Each of the more posteriorly located neurons has a process descending to the thoracic ganglion (Fig. 2C, arrows), where it arborizes in the first and second thoracic neuromeres. Both pairs of neurons and their midline processes are identifiable in third-instar larvae. The descending processes of the more posterior cells start growing at around 15 hours APF. Staining of all midbrain neurons is very reliable at all pupal stages and in adult flies.

Thoracic expression

Most neurons that express *tau-lacZ* are located in the mesothoracic neuromere; several of them can be identified (Fig. 2A,D). The most prominent neuron stained is the TTMn, the identity of which has been confirmed by HRP backfilling of the muscle it innervates and subsequent X-Gal staining (Fig. 2E). *tau-lacZ* expression in the TTMn cannot be observed reliably before 5-6 hours APF but is reliably detected at all subsequent pupal stages and in adults. A more detailed description of its development, produced by intracellular staining with Lucifer Yellow, is given below.

The soma positions of a lateral cluster of four cells (Fig. 2D, black arrows) and one very large dorsomedial cell body, which express *tau-lacZ* (Fig. 2F), makes it likely that these are the DLMns (Coggshall, 1978; Fernandes and Keshishian, 1998; Ikeda and Koenig, 1988; Sun and Wyman, 1997). Verification of the identity of the large cell body was achieved by simultaneous X-Gal staining and HRP muscle backfills from DLM5 (data not shown). Confirmation of the identity of DLMn1-4 is more difficult, because several other neurons are also stained in this region and their dendritic arborizations cannot be easily distinguished. However, X-Gal staining of whole adult flies does show staining of axons innervating all six DLMs (Fig. 2G). *tau-lacZ* expression within DLMn5 can be found consistently as early as 20 hours APF. Expression in the DLMns1-4 is seen at stages from 22 hours APF to adulthood, but is not very reliable and often only 2-3 neurons of the cluster show strong expression.

More variable *tau-lacZ* expression is seen in at least five neurons from about 16 hours APF (Fig. 2A,D). One, with a cell body located at the border between the pro- and mesothoracic neuromere (Fig. 2D, white arrows), sends processes to the midline just anterior to the characteristic TTMn dendritic bend. This cell is probably also stained in the *Gal4* enhancer-trap line A307 (Allen et al., 1998; Phelan et al., 1996). Two cell bodies are located within the metathoracic neuromere (Fig. 2D, arrowheads) and also send midline processes towards the TTMn bend. A fourth large cell body (stars) sends processes towards the midline. The fifth cell body, which is probably the PSI (Fig. 2D, asterisk), is located medial to the TTMn cell body and projects to the midline. The PSI on the right side of Fig. 2D probably lies beneath the large cell body marked with a star.

In third-instar larvae and early pupae (up to 30 hours APF) a number of segmentally repeated neurons, which are probably motorneurons, express *tau-lacZ* in all three thoracic segments (Fig. 2H, white arrowheads). These labelled neurons are situated near the dorsal midline, although ventral midline cells are found (not shown). Additionally, a variable number of

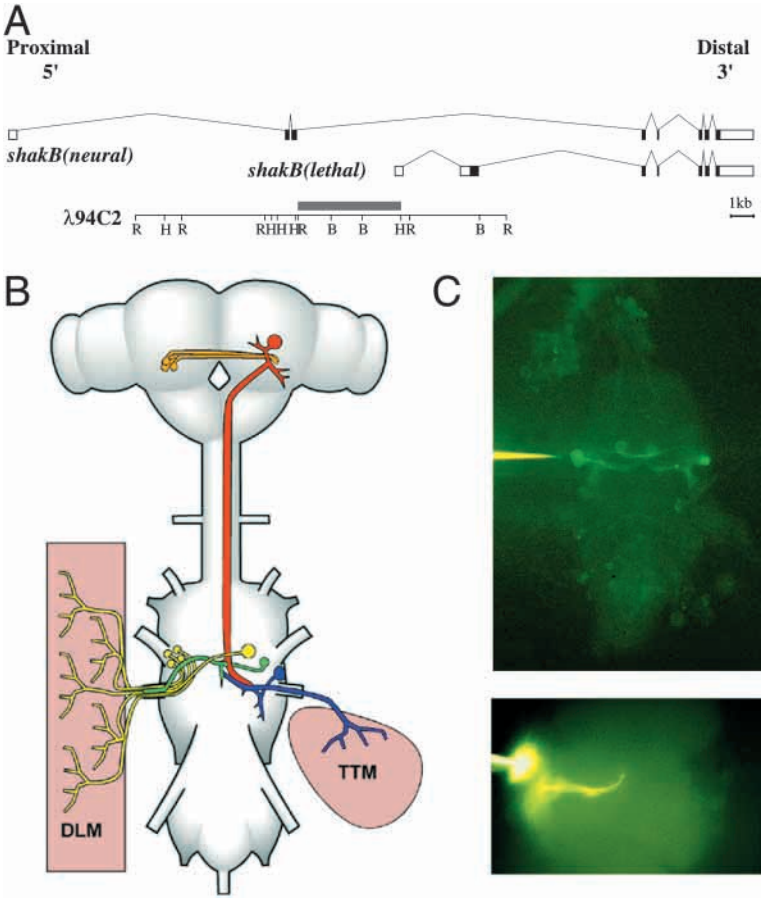

Fig. 1. (A) Position of the 4.1 kb genomic fragment used to construct *shakB(lethal)-Gal4*. Two *shakB* transcripts are shown as reference; white boxes are untranslated exons, black boxes indicate coding region and introns are marked as lines. Genomic clone λ94C2 is shown mapped onto the *shakB* locus. The extent of the 4.1 kb *Eco*RI-*Hin*dIII fragment used to make the reporter construct *plethal-Gal4* is shown as a grey box. Restriction sites are marked: R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI. (B) Schematic representation of the GFS (modified from Blagburn et al., 1999); each neuron shown has a contralateral homologue. In the brain, the GF (red) is contacted by terminals of at least three contralaterally situated Giant Commissural Interneurons (orange). In the mesothoracic neuromere, the GF synapses directly with the TTMn (blue) and indirectly, via the PSI (green), with the DLMns (yellow). The DLM and TTM are shown in longitudinal and transverse section respectively. (C) A *tau-GFP* reporter labels cells expressing the *shakB(lethal)-Gal4* construct (top) and allows subsequent penetration of the TTMn with a Lucifer Yellow filled electrode (bottom).

smaller neurons including some ventral unpaired motorneurons (VUMs), can be detected in the thoracic neuromeres, depending on developmental stages and incubation times of the X-Gal reaction.

Abdominal expression

tau-lacZ expression in a segmentally repeated set of neurons is first seen in third-instar larvae. In each neuromere, a number of paired neurons stain near the midline, several at the dorsal surface of the ganglion (Fig. 2H, black arrowheads) and one to two near the ventral surface (Fig. 2I, black arrowheads). They all send axons to the periphery, and so they are probably motorneurons. In the first four abdominal neuromeres, two pairs of laterally situated motorneurons are labelled up to 30 hours APF (Fig. 2H, arrows); we were not able to determine their dendritic anatomy. From 30 hours APF onwards, abdominal *tau-lacZ* expression begins in several motor- and/or interneurons; these cell bodies are not organized in an obvious segmentally repeated pattern (Fig. 2A).

Development of TTMn dendrites

Intracellular Lucifer-Yellow fills provide much better resolution of TTMn morphology at different developmental stages than can be determined by *tau-lacZ* or *tau-GFP* reporters (Figs 1B, 2, 3). In addition, this technique can be used to reveal dye coupling between the TTMn and other neurons of the GFS (Phelan et al., 1996). In adult flies, the TTMn cell body is located ventrolaterally in the mesothoracic neuromere (Fig. 2A,D,E). The neurite extends from the cell body in a dorsomedial direction and gives rise to two prominent dendritic branches, one of which extends posterioventrally and the other one in a ventromedial direction. The posterior dendrite bifurcates to produce two major branches with secondary branching. The medial dendrite grows straight to the midline, where it bends and contacts the ipsilateral GF and PSI (Fig. 2E, arrow; King and Wyman, 1980).

GFP expression in the TTMn cell body begins at approximately 5-6 hours APF. The first successful impalement of TTMn cell body with a Lucifer Yellow filled electrode was achieved at 9 hours APF (Fig. 3A). At this time, the axon has extended into the periphery (Fig. 3A, arrowhead) and the medial dendrite has a large growth cone, which is already near the midline (Fig. 3E). It reaches the midline no later than 16/17 hours. About 15-20 μm lateral to the midline, the dendrite turns anteriorly to form its characteristic bend (Fig. 3F, arrow). From 14 to 28 hours APF, the distal part of the medial dendrite has a growth-cone-like structure, with fine filopodia found mainly at the distal tip of the dendrite and more proximally at the region of the bend (Fig. 3F,G). The growth cone was never observed to cross the midline. Shortly before onset of coupling to the PSI (see below) the distal tip of the medial dendrite bifurcates and forms two short branches with separate growth cones (Fig. 3G).

At around 15 hours APF, the posterior dendrite diverges from the major dendrite to grow in a posteriolateral direction (Fig. 3B, arrowhead). By about 20 hours APF, this dendrite has turned more posteriorly, and it branches at 25 hours APF to give rise to the two main secondary neurites (Fig. 3C, arrow). Both form small arborizations from the branching point to the posterior border of the mesothoracic neuromere, which they reach at approximately 45 to 50 hours APF (Fig. 3D). At later stages, a general straightening of the cell body-neurite-posterior dendrite axis occurs.

Development of intercellular communication in the GFS

Lucifer Yellow is known to pass through gap junctions formed between neurons of the GFS (Phelan et al., 1996). We analysed

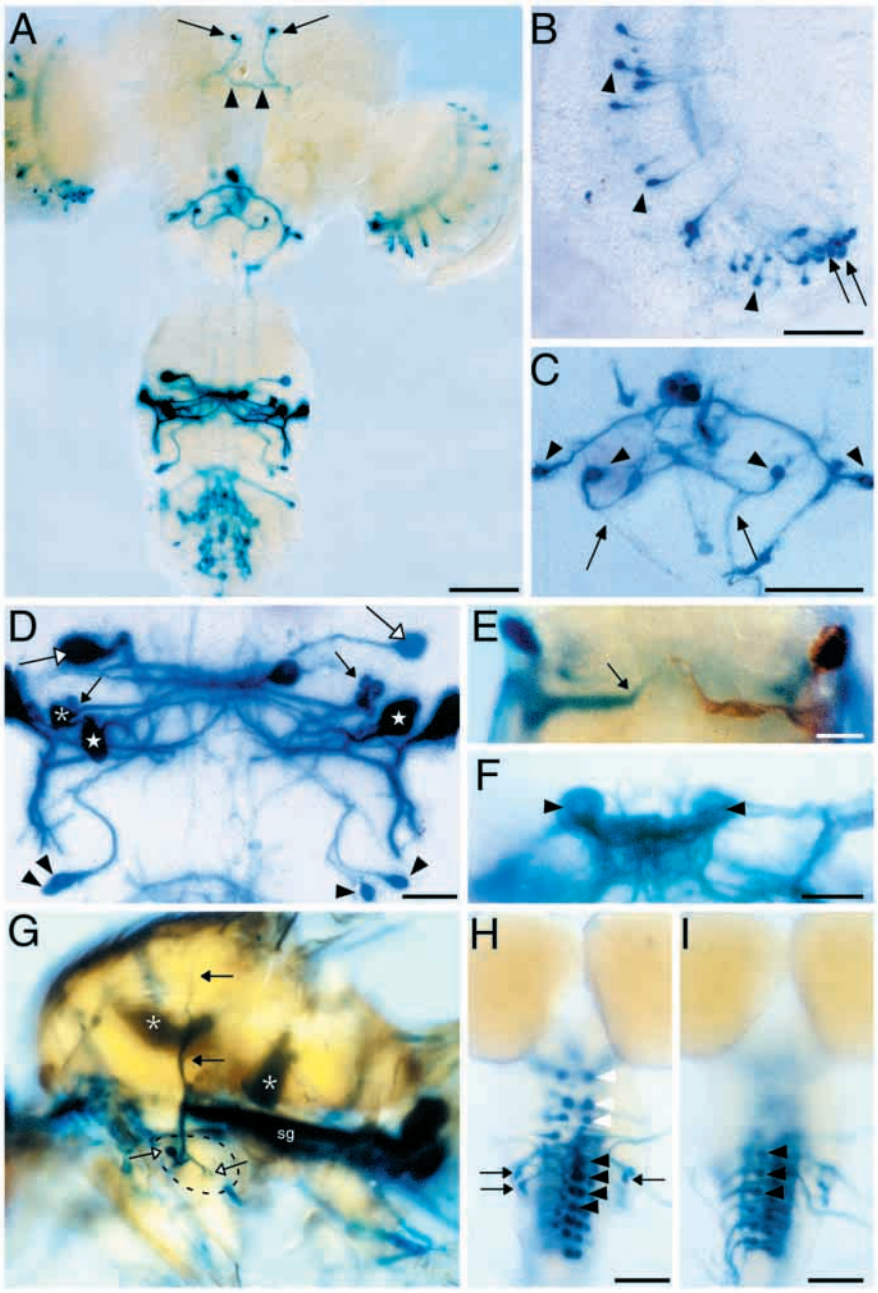

Fig. 2. Relatively few neurons express *tau-lacZ* in *shakB(lethal)-Gal4* flies. (A) Whole mount of a pupal brain and thoracic nervous system at 52 hours APF. Several neurons in the optic lobes, a pair of cells in the midbrain (arrows) and two pairs of cells in the suboesophageal ganglion are stained. Medial branches of the midbrain neurons can be seen clearly (arrowheads), their lateral branches are not visible in this photograph. Thoracic expression of *tau-lacZ* is strongest in the mesothoracic neuromere, where several neurons of the GFS are stained. Some neurons are stained in the abdominal neuromeres. (B) Two types of neuron in the optic lobes express *tau-lacZ*. Cells of the first type are spaced throughout the medulla (arrowheads). Cell bodies of the second type are clustered in the cortex of the posterior optic lobe (arrows). (C) Two pairs of neurons express *tau-lacZ* in the suboesophageal ganglion (arrowheads). Both have medially projecting processes, and at least one pair has additional descending processes (arrows). (D) Higher power view of all mesothoracic neurons shown in A. A group of 4 small cells is likely to be the DLMns 1-4 (black arrow). DLMn 5 is out of focus. One cell is probably the PSI (asterisk), which on the right side lies beneath the large cell body of another neuron (star). The neuron marked with an open arrow has probably previously been labelled in enhancer trap line A307 (Phelan et al., 1996). Two cell-bodies are located in the metathoracic neuromere (arrowheads) and also send midline processes towards the TTMn bend. (E) Double staining of the TTMn with X-Gal (left) and HRP, backfilled from the TTM (right), showing the characteristic bend fo the TTMn (arrow). (F) Cell bodies (arrowheads) and dendritic arborizations of the DLMn5. (G) X-Gal staining of a whole-mount fly. Anterior is to the left. Most prominent *tau-lacZ* expression is in the salivary glands (sg). Weaker expression is seen in a few muscles (asterisks). The thoracic ganglion is situated ventrally (dashed line), with cell body and posterior dendrite of the TTMn (white arrows) in focus. All six dorsal longitudinal muscles are innervated by stained axons which project dorsally after leaving the CNS. Arrows indicate two of these axons leaving the major fascicle to innervate their muscles. (H,I) Several pairs of segmentally repeated motorneurons express *tau-lacZ* in the thoracic and abdominal neuromeres of larvae and early pupae. The example shown is 3 hours APF. At least two pairs of neurons are located near the dorsal midline in the thoracic (H, white arrowheads) and abdominal (H, black arrowheads) neuromeres, one to two pairs can be found near the ventral midline (I, arrowheads). In addition, in abdominal segments 1 to 4, ventrolaterally located neurons express *tau-lacZ* (H, arrows). Scale bars, A,H,I, 100 μm; B,C, 50 μm; D-F, 25 μm.

the development of dye coupling between the TTMn and other neurons of the GFS by injecting the TTMn with Lucifer Yellow at different pupal stages.

The PSIs are the first neurons to dye-couple with the TTMn (Fig. 3H). This dye coupling does not appear until 28-32 hours APF, despite the fact that the growth cone has reached the midline by 14 hours APF. During the period of 14-28 hours APF, the anterior extension of TTMn's growth cone along the midline does not appear to be related to time after puparium formation; this indicates either differences between individuals or dynamic extension and retraction of the growth cone. By 28 hours APF, the filopodia of the TTMn have withdrawn, the growth cone has collapsed, and the medial dendrite appears smooth. At this and at all subsequent stages, the ipsilateral PSI is more strongly coupled to the injected TTMn than is the contralateral PSI. Transjunctional dye-fillings show that the PSI has extensive ipsilateral dendritic branching near the midline (Fig. 3H, arrow) and an axon projecting to the contralateral posterior dorsal mesothoracic nerve (Fig. 3H, arrowhead), the point at which it forms chemical synapses with the DLMns (Egger et al., 1997).

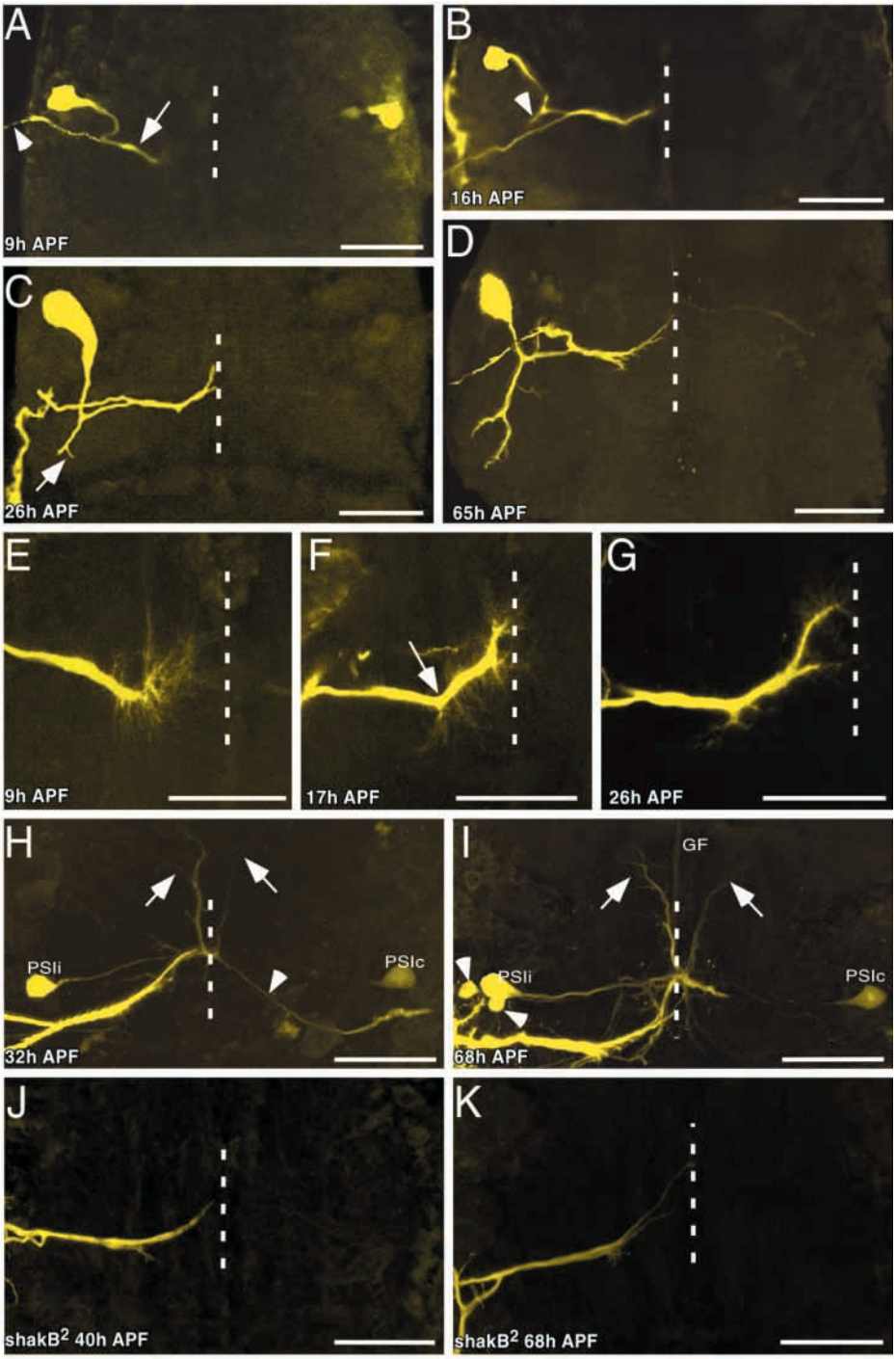

Fig. 3. Lucifer-Yellow fills of TTMn reveal its morphogenesis and synaptogenesis during metamorphosis. (A,E) At 9 hours APF, filopodia from the medial dendrite (arrow) have nearly reached the midline (dashed line). The axon (arrowhead) is already projecting into the periphery. The second cell body in A is an aborted fill of the contralateral TTMn. Anterior is to the top. (B,F) At 16/17 hours APF the medial dendrite has reached the midline and has already formed its bend (arrow in F), with filopodia emanating from all of the dendrite medial to the bend. The posterior dendrite has started to form (arrowhead in B). (C,G) At 26 hours APF, the posterior dendrite has bifurcated (C, arrow) and the medial dendrite has split near the midline. Filopodia are now mainly found at the two tips of the medial dendrite and at the position where this dendrite bends anteriorly. (D) By 65 hours APF, the TTMn has largely achieved its adult morphology. By this stage, TTMn is dye-coupled to some other neurons of the GFS, but levels of focus, showing dye coupling, were not included in the composite micrograph. (H) At 28-32 hours APF, both ipsilateral (PSIi) and contralateral (PSIc) peripheral synapsing interneurons become dye coupled to TTMn. Dendrites of the PSIs already show first order branches (arrows). The axon of PSIi exits the CNS contralaterally (arrowhead). (I) The ipsilateral GF dye couples at around 42 hours APF, and from 60 hours APF more interneurons become dye coupled (arrowheads). Note that the medial dendrite of TTMn shows a second bout of sprouting and that the PSI dendritic branching is more extensive at 68 hours APF than it is at 32 hours APF (arrows). (J,K) In *shakB*^{2} animals no dye-coupling is visible between TTMn and other neurons of the GFS at 40 hours (J) and 68 hours (K) APF. The second wave of sprouting of the medial dendrite is also visible between 60 to 90 hours APF in these *shakB*^{2} animals (compare K to I). Scale bars, A-D, 50 μm; E-K, 25 μm.

Dye coupling between the TTMn and the ipsilateral GF was first seen at about 42 hours APF, however dye filling from the TTMn was always much weaker to the GF than it was to the ipsilateral PSI (Fig. 3I). The medial dendrite of TTMn has a second phase of sprouting between 60 and 90 hours APF (Fig. 3D,I), which may indicate a second phase of synaptogenesis. Indeed from about 60 hours onwards, additional neurons with cell bodies near the ipsilateral PSI become dye-coupled to the TTMn (Fig. 3I, arrowheads). These cells, which are probably the same as those observed after dye filling the GF (Phelan et al., 1996), are probably interneurons because we never detected any peripheral axons in addition to those of TTMn and PSI. We have never seen dye entering the contralateral TTMn or contralateral GF, from the injected TTMn, at any pupal stage.

It has been reported previously (Blagburn et al., 1999; Phelan et al., 1996) that formation of the GF-TTMn electrical synapse depends on activity of the *shakB* locus. We confirmed these data by dye-filling the TTMn in *shakB*^{2} pupae. In all of these animals, dye coupling of the TTMn to the GF is never established (Fig. 3J,K). Furthermore, TTMn-PSI's dye coupling was also eliminated, indicating that formation of the electrical synapses between these neurons also requires

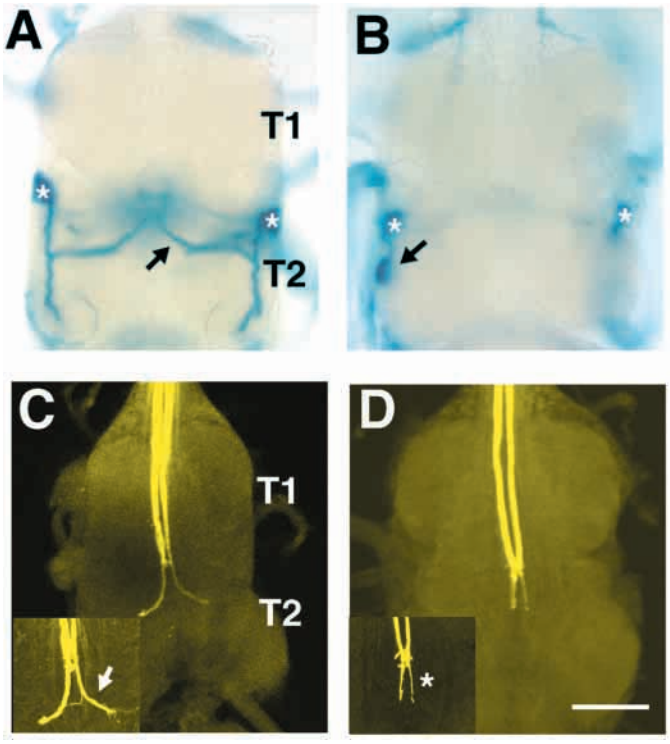

Fig. 4. GF thoracic morphology is dependent on the presence of the TTMn medial dendrite. (A) The wild-type adult morphology of both TTMns in the second thoracic neuromere (T2) is revealed by *tau-lacZ* expression. Each cell body is marked with an asterisk, the right-hand medial dendrite is marked with an arrow. (B) Expression of *Dcdc42* arrests the growth of the medial dendrite of TTMn. The cell body (asterisk) and truncated lateral neurite (arrow) are visible. (C) GF morphology in control flies revealed by injecting each GF axon, to show the characteristic bends in T2. Insert shows a higher resolution view of the same preparation. (D) In the absence of the TTMn medial dendrites, the GF axons remain stranded at the midline. Insert shows thin, aberrant processes (asterisk). Scale bar, 50 μm.

ShakB(neural) function. We were unable to detect any obvious differences in the timing of development or the final pupal morphology of TTMn in wild type and *shakB*^{2} mutants (*n*=26). In addition, the TTMn in the mutant displays a very similar second stage of sprouting at 60-90 hours APF (Fig. 3K).

Development of the GF in the absence of the TTMn medial dendrite

Our results imply that the TTMn medial dendrite forms its characteristic bend before making contact with the GF. To determine whether formation of the distinctive GF bend is dependent on the presence of the TTMn, we arrested the outgrowth of the TTMn medial dendrite by targeted expression of an activated form of the small GTPase, Dcdc42. Constitutively active mutant forms of both Dcdc42 and Drac1, another small GTPase, have been shown to block the outgrowth of neurons in the *Drosophila* CNS (Luo et al., 1994; Kaufmann et al., 1998; Allen et al., 2000).

In all flies carrying *shakB(lethal)-Gal4, UAS-Dcdc42(V12)* and *UAS-tau-lacZ*, the reporter showed the presence of the TTMn cell bodies, but much reduced or absent medial dendrites (*n*=22; Fig. 4B). All controls, that only lacked *UAS-Dcdc42(V12)*, showed wild-type TTMn morphology (*n*=30; Fig. 4A). To determine the morphology of the GF in flies lacking a normal TTMn medial dendrite, the GF was filled with Lucifer Yellow by impaling its axon in the cervical connective. This revealed a consistent and distinctive defect; the GF always lacked its normal bend (*n*=18; Fig. 4D). Interestingly, fine processes were always seen emanating from the distal tip of the GF, which often projected laterally or extended towards the metathoracic neuromere (Fig. 4D, asterisk). The GF in control animals produced its normal bend (*n*=15; Fig. 4C).

DISCUSSION

The TTMn is probably an embryonic neuron

In general, there are two discrete periods of neurogenesis in holometabolous insects. During embryogenesis, the neurons that constitute the larval CNS are born, while a second round of neurogenesis at larval and early pupal stages supplies additional adult neurons (Truman and Bate, 1988; Truman et al., 1993). BrdU labelling strongly suggests that the GF is born during embryogenesis, the first period of neurogenesis, but delays axogenesis until the very end of larval development (Allen et al., 1998), reaching the mesothoracic neuromere in the first 25 hours APF (Phelan et al., 1996).

Our reporter construct only expressed reliably in the TTMn from 5 to 6 hours APF onwards and so provides no data on TTMn's birthdate. However, like the GF, indirect evidence suggests that TTMn is also an embryonic neuron that delays morphogenesis until the pupal stage. Previous studies using BrdU incorporation suggest that all large motorneurons (such as TTMn) are born early in embryogenesis (Truman and Bate, 1988). For example, leg motorneurons are probably present in the larva, and they appear to first extend their axons into the leg imaginal disc to supply the developing disc musculature early in metamorphosis (M. Bate, personal communication). In accordance with this, the TT muscle is known to form de novo early in metamorphosis from myoblasts accumulating at the leg imaginal disc (Fernandes and Vijayraghavan, 1993), and we observe that the TTMn is already projecting its axon into the periphery as early as 9 hours APF.

Ultrastructural studies have shown that the peak of larval dendrite degeneration in *Drosophila* CNS occurs between 12 and 18 hours APF (Singh and Singh, 1999). Data on identified *Drosophila* motorneurons undergoing metamorphosis is somewhat limited, but Fernandes and Keshishian (1998) have shown that DLMn5 begins producing adult dendritic branches at approximately 24 hours APF. In our work we never detected any dendritic branches at the early stages of metamorphosis from 9 to 24 hours APF, other than the outgrowing medial dendrite. The most parsimonious explanation of these observations is that the TTMn has no larval dendritic branches, and hence no larval function. Taken together, these results indicate that GF and TTMn develop their central arborisations only shortly before the GFS is active in adult flies.

Formation of GF's characterisitic bend is a fasciculation event

The first physical encounter between the GF and the TTMn has been under intense scrutiny in the literature. Allen et al. (1998) have proposed that the GF-TTMn bend is formed after an

initial right angled contact between the GF and TTMn at the midline and that the bending of both neurons is a result of subsequent morphological movements of the ganglion. The Lucifer-Yellow fills of the TTMn described in the present study show that its bend starts forming as early as 14 hours APF. At this time the dendrite has a large and dynamic growth cone which makes two 45 degree turns, the first of which is at least 15 μm from the midline. The GF axons are confined to the midline at this stage, and we conclude therefore that the TTMn makes its first bend independent of the GF.

We have shown that the subsequent 'bending' of the GF is dependent on the presence of the TTMn dendrite by specifically blocking neurite outgrowth of the motorneuron. The arrest of the GF at the midline, and the appearance of fine exploratory processes from the distal tip of the GF axon, indicate that the TTMn normally provides the signals for bending and synaptogenesis. This is consistent with the 'bendless' phenocopy observed when retrograde signalling is blocked in the GF despite the presence of a normal TTMn (Allen et al., 1999). We conclude that the GF bend is not caused by major morphological movements of the ganglion (Allen et al., 1998), but is a result of its fasciculation with the curved TTMn. However, the GF axon does appear to stop in the correct position in the mesothoracic neuromere in the absence of the TTMn dendrite; this indicates that the developing axon receives information from other cells in the ganglion and is not wholly reliant on the TTMn to prevent overgrowth.

Electrical synapses are formed in a defined temporal sequence

Previous studies demonstrated that several of the synapses made between neurons of the GFS in *Drosophila* are electrical (Blagburn et al., 1999; King and Wyman, 1980; Phelan et al., 1996; Tanouye and Wyman, 1980). The first point at which Lucifer Yellow can be reliably injected into the axon of the GF is 45 hours APF, and at this stage it is already coupled to the TTMn (Phelan et al., 1996). Three hours later, the GF is coupled to the PSIs. In contrast, the methodology used here allows the formation of synapses by the TTMn to be investigated at earlier stages of pupal development. Our results confirm the timing of gap junction formation between the TTMn and the GF (about 42 hours APF), but also show that the TTMn is coupled to the PSI significantly earlier in development, at about 28-32 hours APF. The TTMn is always more strongly dye-coupled to the ipsilateral than to the contralateral PSI. The morphological studies of King and Wyman (1980) show that the TTMn only comes into contact with the ipsilateral PSI, and that the two PSIs come into extensive cell-cell contact at the midline. The most likely scenario therefore to explain the dye-coupling we see is that Lucifer Yellow passes from the TTMn into the ipsilateral PSI and then into the contralateral PSI. We assume that, after 42 hours APF, the dye-filling into the ipsilateral GF is weaker than into the ipsilateral PSI because the larger volume of the GF dissipates the Lucifer Yellow.

After their mutual contact is established, both the TTMn and the PSI continue growing neurites in other regions. The anterior projecting arborizations of the PSI achieve their adult morphology at approximately 40-45 hours APF, and the posterior dendrite of the TTMn stops growing just after 45-50 hours APF. To date, nothing is known about other synaptic partners of the PSI or TTMn, but clearly their aborizations indicate either involvement in other neuronal circuits or the existence of so far unknown components of the GFS. Interestingly, from 60 to 90 hours APF, the TTMn medial dendrite shows a second phase of sprouting activity. These sprouts may indicate that the TTMn is at that time searching for yet other synaptic partners in the midline region and we observed additional neurons becoming dye-coupled to the TTMn during this period. Sprouts from the medial dendrite of TTMn are not seen in adults.

Do electrical synapses formed by the TTMn play a developmental role?

Evidence for the possible involvement of transient gap-junctional communication in axon guidance and synaptogenesis comes from both vertebrate and invertebrate systems (Bentley et al., 1991; Chang et al., 1999; Lakes-Harlan and Pollack, 1993; Taghert et al., 1982; Wolszon et al., 1994). Though we did not see any transient dye-coupling from TTMn to other cells in the CNS during its development, the establishment of permanent electrical synapses might, in principle, allow developmentally important ions and/or small molecules to pass between cells. However, we were unable to detect any obvious developmental differences in TTMn morphology in wild type and *shakB*^{2} mutants during pupation. Though our observations are limited to the confocal-microscope level, we do note that in adult *shakB*^{2} mutants, the ultrastructure of the chemical synapses between GF and TTMn (Blagburn et al., 1999) and TTMn physiology (Baird et al., 1990) also appear normal. As dye coupling between the TTMn and neurons of the GFS is eliminated in *shakB*^{2} mutants, these gap junctions appear to play no obvious roles in these aspects of TTMn development. Of course, it is possible that gap-junctional communication, not mediated by products removed by the *shakB*^{2} mutation and undetectable by dye-coupling, does play a role in the development of these aspects of TTMn.

Some features of TTMn are altered in adult *shakB*^{2} mutants. Baird et al. (1993) reported a significant reduction in the diameter and anteroposterior extent of the medial dendrite of TTMn along the midline of adult *shakB*^{2} flies (our study only examined TTMn morphology in pupal *shakB*^{2} flies). Whether the anatomical differences observed by Baird et al. (1993) are a direct result of a lack of gap-junctional communication between TTMn and appropriate cellular partners during development remains to be determined.

What is the role of *shakB(lethal)* in the GFS?

The *shakB(lethal)-Gal4* construct contains 4.1 kb of upstream regulatory region of *shakB(lethal)*, including its promoter. A comparison of reporter gene expression in *shakB(lethal)-Gal4* flies and wild-type *shakB(lethal)* expression, as revealed by in situ hybridization, indicates that *shakB(lethal)-Gal4* flies do indeed drive a reporter gene in neurons which normally transcribe *shakB(lethal)* (Crompton et al., 1995). Examples of such neurons (identified by their cell body positions) are the two pairs of neurons in the suboesophageal ganglion and cells in the optic lobe. In situ hybridization also revealed *shakB(lethal)* expression in several neurons of the mesothoracic neuromere, with their cell bodies located in very similar positions to neurons that express *tau-lacZ*. The fact that *tau-lacZ* expression in many embryonic mesodermal cells (unpublished observations) reflects that of *shakB(lethal)* (Crompton et al., 1995) also supports the idea that the 4.1 kb

genomic fragment used in this study confers a pattern of expression that represents at least a subset of the wild-type expression pattern of *shakB(lethal)*. However, the abdominal neurons that express *tau-lacZ* did not appear to express *shakB(lethal)* in the previous in situ hybridization study (Crompton et al., 1995). This could reflect insufficient resolution by in situ hybridization. Alternatively, the regulatory region of *shakB(lethal)* that we have selected is either incomplete or incorporates elements that are not normally utilised for the expression of *shakB(lethal)*.

Given that *shakB(lethal*) is expressed in some elements of the GFS (TTMn, DLMns and possibly the PSIs), but that the dye-coupling reported in this work requires ShakB(neural) protein(s), what requirement could there be for ShakB(lethal)? Certainly ShakB(lethal) and one or more of the ShakB(neural) proteins might form heterotypic gap junctions – in that case the loss of ShakB(neural) in the GF would necessarily result in loss of dye coupling between GF and TTMn. In addition, ShakB(lethal) protein can form homotypic gap junctions (Phelan et al., 1998b) and the work of Koenig and Ikeda (1983) suggests that the activity in DLMns 1-4 is synchronised by gap junctions; these might be formed by ShakB(lethal) protein. ShakB antibodies raised to a common region of ShakB(neural) and ShakB(lethal) show the strongest staining in the anterior mesothoracic neuromere at the site of synapse formation between the GF and TTMn/PSIs (Phelan et al., 1996). ShakB expression in mutants that lack neural proteins is almost completely eliminated, but persists at the site of the GF/PSI and TTMn/PSI synapses, suggesting expression (and possibly involvement) of ShakB(lethal) at these synapses. Nevertheless, this very restricted expression of the ShakB(lethal) protein as revealed with an antibody is not in accordance with the widespread expression of the corresponding transcript, as shown by in situ hybridization. One possible explanation for this is that ShakB(lethal) may be detected at this site only because synapses of several neurons converge here, thus increasing the local protein concentration to detectable levels. As flies mutant for *shakB(lethal)* are not viable, it has not been possible to determine the role of *shakB(lethal)* at the site of the TTMn/GF synapse directly. Techniques such as dsRNA interference (Fire et al., 1998; Marie et al., 2000) might in future provide the necessary tools to further investigate any developmental and physiological roles of the gap junctions at this highly accessible synapse.

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