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Patterning Sensory Axon Projections in the *Drosophila* Embryonic Nervous System

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ABSTRACT

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PATTERNING SENSORY AXON PROJECTIONS IN THE DROSOPHILA EMBRYONIC NERVOUS SYSTEM

by Louise Matilda Christie Block

Sensory neurons make specific axonal projections in the CNS according to their position and modality. The genetic specification of position and sensory identity therefore determines where an axon terminates in the CNS. This process is central to the generation of an ordered CNS. The aim of the work presented here was to examine the mechanisms that control the translation of positional information and sensory modality into patterns of central projections. Although many genes involved in determining sense organ type have been identified, little is known regarding the specification of positional values within the sensory nervous system. One possibility is that the segmentation genes that pattern the early embryo also provide positional information within the developing sensory nervous system.

A detailed analysis of segmentation gene expression within the sensory nervous system was conducted to create a map of gene expression within each sensory neuron. This revealed that very few of these genes are re-expressed in the embryonic sensory nervous system at any stage during its development. However *armadillo*, *engrailed* and *runt* were identified as candidate genes for specifying cell fate in the sensory nervous system. In order to test the role of these candidate genes in determining cellular identity during development two different misexpression approaches were taken. First, an existing collection of P[GAL4] enhancer-trap lines was screened to identify lines which would enable ectopic gene expression to be driven in subsets of sensory neurons. Few useful drivers were identified due to the late onset of GAL4 activity in the majority of lines screened. The pan-neural elav-GAL4 enhancer-trap line was also used to express candidate genes throughout the nervous system. For *engrailed* this resulted in disruptions to nervous system development consistent with a role in specifying cell fate, indicating that *engrailed* is likely to play a role in nervous system development.

A second approach was to develop techniques that would enable gene expression to be targeted to individual sensory neurons. The potential of laser-induced heatshock as a means of activating gene expression in single cells and of the MARCM technique, which enables mosaic analysis to be conducted in a cell-specific manner, were explored. While constitutive activation of gene expression was successfully achieved, laser induced gene expression occurred at such a low rate as to be impractical when compared to the amount of effort required to generate laser treated material. The MARCM technique provided a means of creating single neuron clones and enabled sensory axons to be visualised in the CNS. and has been used to characterise the normal pattern of central projections for each embryonic sensory neuron in the larval CNS. This analysis will provide a framework in which the MARCM technique can be used to examine the function of *engrailed* in specifying axonal morphology.

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Someone once told me that the questions that drive you in later life are often the ones that you work on during your PhD. I would therefore like to also thank Dave for initiating an interest in neural circuit formation and axon patterning which has developed into a passion.

Introduction

Santiago Ramón y Cajal revolutionised neuroscience with the discovery that the nervous system is composed of billions of separate nerve cells, rather than a continuous network of filaments as had previously been thought. This led to the realisation that the basic units of the nervous system were individual neurons, and this conclusion is the founding principle of nervous system organisation. Cajal's observations served to focus attention on the organisation of the nervous system being key to understanding its function.

The nervous system comprises a plethora of neuronal cell types organised into highly ordered networks of synaptic connections. These precise patterns of neuronal circuitry are laid down during development and for an organism to be able to sense and respond to its environment it is absolutely essential that these neurons become correctly assembled into functional circuits. Ordered arrays of sensory neurons, such as the somatotopic representations of the body in the human somatic sensory cortex, are central to the assembly of a functioning nervous system yet little is known regarding the mechanisms that establish this order. Much of our understanding of the development of the nervous system has come from the study of the comparatively simple nervous systems of invertebrates, particularly insects. Here too sensory neurons project to the central nervous system in an orderly manner, and provide an invaluable model in which to address the mechanisms that underlie the formation of sensory maps.

Several features have made the insect nervous system especially suitable for the analysis of sensory projections. Firstly, the generation of the sensory nervous system and the central nervous system (CNS) are spatially independent processes, permitting the formation of connections between the two to be examined. Secondly, sensilla occur in stereotyped arrays in the periphery and appear consistently in the same positions, meaning that individual neurons can be repeatedly identified. In addition, transplantation of insect epidermis is comparatively easy and is accompanied by vigorous regeneration of sensory cells.

1.1 Organisation of insect sensory systems

Classical anatomical techniques reveal three main regions of neuropil in each insect ganglion: ventral neuropil, intermediate neuropil and dorsal neuropil (Strausfeld, 1976). Examination of sensory afferents in a wide range of insects has shown that different types of sense organ arborise within different layers of the CNS (e.g. Murphey et al., 1985; Murphey et al., 1989a; Pflüger et al., 1988), and that these projection areas correspond to the regions of neuropil

revealed by classical anatomical techniques (Murphey et al., 1985). Each ganglion is organised similarly (Murphey et al., 1985) and this organisation is also highly conserved across species ranging from *Drosophila* to the grasshopper.

Modality specific projections in both *Phormia* and *Drosophila*, for example, are virtually indistinguishable (Murphey et al., 1989a). Leg tactile neurons innervate a ventral region of leg neuropil whereas hair plate axons, which convey proprioceptive information, innervate intermediate neuropil (Murphey et al., 1989a). The projection of leg tactile neurons to ventral leg neuropil is conserved in crickets (Johnson and Murphey, 1985) and locusts (Burrows and Siegler, 1984) and remains distinct from hair plate axons, which also segregate to intermediate neuropil in the locust (Pflüger, 1980). Proprioceptors occupy an intermediate region of neuropil in flies (Merritt and Murphey, 1992; Murphey et al., 1989a) crickets (Hustert, 1978), and locusts (Pflüger, 1980; Pflüger et al., 1988). In *Drosophila* and *Phormia*, where the sensory afferents of gustatory neurons have been described (Murphey et al., 1989a), these axons also innervate the ventral neuropil but aborise within a distinct medioventral region of this neuropil. Likewise, chemosensory neurons occupy a discrete sub-division of ventral neuropil in locusts (Newland and Burrows, 1994; Pflüger et al., 1988). The modality specific segregation exhibited by chemosensory, tactile, and proprioceptive axons within insect ganglia is shown schematically in Figure 1.1.

Within each projection region axons are often arrayed topologically, as illustrated in Figure 1.1. Somatotopic (Murphey et al., 1980; Murphey et al., 1989b; Newland, 1991), retinotopic (Braitenburg, 1972; Horridge and Meinertzhagen; 1970), and tonotopic (Oldfield, 1983; Römer, 1983) maps have all been described. Retinotopic mapping refers to the orderly projection of retinular fibres within the visual processing centres of the brain, the optic lobes. The spatial relationships between retinal cells are retained within the optic lobes, thus providing a central representation of visual space. In *Drosophila*, for example, each ommatidia contains 8 photoreceptor neurons (R1-8) which project retinotopically to different layers of the optic lobe (Meinertzhagen and Hanson, 1993). R1-6 terminate in the lamina, where they form a 'neurocrystalline' array of synaptic cartridges, while R7 and R8 project to separate layers of the medulla.

Tonotopic mapping, where sensory afferents are organised according to their sensitivity to different frequencies of vibration, is exemplified by the cricket auditory system. Cricket tympanal organs, the crista acoustica, comprise about 40 chordotonal receptor cells and are located in the tibia of the foreleg. Each receptor cell is tuned to a particular frequency

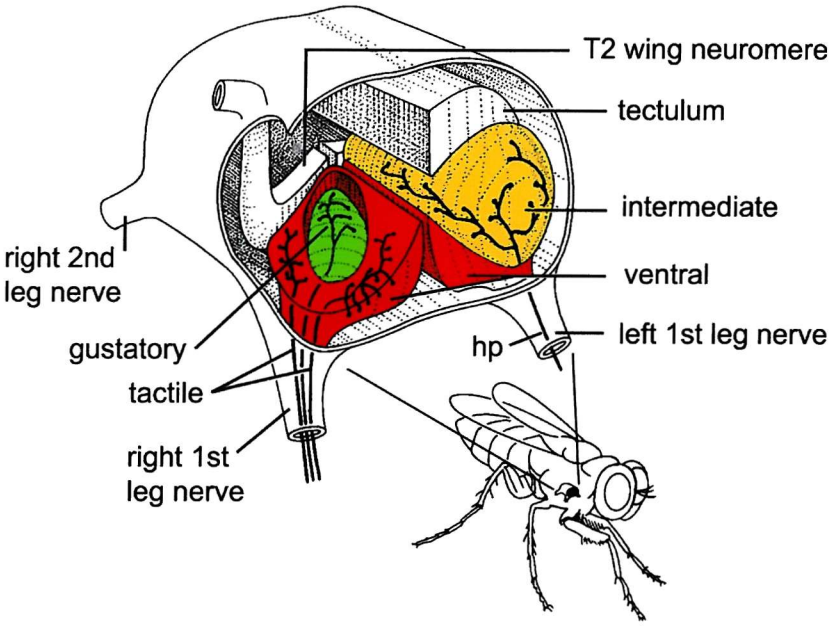


Figure 1.1 Central organisation of sensory projections in flies. Summary diagram illustrating the modality specific and topological organisation of sensory projections within the fly ganglia. Hair plate afferents (hp) project to the intermediate neuropil. Chemosensory afferents project to a medio-ventral neuropil (gustatory). Tactile afferents project to the ventral neuropil and surround the gustatory afferents. Somatotopic organisation is illustrated in the ventral neuropil, where two tactile neurons are shown. Tactile neurons on the anterior of the leg project to the anterior tactile neuropil while tactile neurons on the posterior of the leg project to the posterior tactile neuropil. The inset indicates the internal position of the depicted ganglia. Figure reproduced from Murphey et al. (1989a).

(Rheinlaender, 1975). Within the crista acoustica receptors are ordered such that proximal and distal sensilla respond to low and high frequencies respectively (Oldfield, 1982). This tonotopic organisation is preserved in the auditory neuropil, where fibres tuned to low frequencies project anteriorly and those tuned to high frequencies project posteriorly (Oldfield, 1983; Römer, 1983; Atkins and Pollack, 1987). The spatial representation of frequency observed in the cricket auditory system is analogous to the organisation of the cochlea and brain in the vertebrate auditory system.

Somatotopic mapping, where sensory afferents form ordered projections according to the relative position of a sensory neuron in the periphery, is illustrated by insect mechanosensory systems. Central projections of tactile hairs on the hind leg of the locust, for example, form a somatotopic map which corresponds precisely to the position of each sensilla, and represents the location of each hair in three dimensions (Newland, 1991). This contrasts with tactile leg afferents in *Drosophila* and the cricket (Johnson and Murphey, 1985; Murphey et al., 1989b), where the somatotopic map is determined by circumferential position. Dorso-ventral position is reflected in the dorso-ventral axis of the central projection region and anterior-posterior position is represented centrally in the medio-lateral axis. The proximo-distal position of each hair is represented along an anterior-posterior central axis.

Mechanosensory afferents from wind-sensitive and tactile hairs form somatotopic maps in the CNS of crickets (Johnson and Murphey, 1985; Murphey et al., 1980) flies (Murphey et al., 1989b), moths (Levine et al., 1985), and locusts (Newland, 1991; Pflüger et al., 1981). Experimental evidence suggests that such precise mapping is not simply a correlate of temporal order of birth, initial axon trajectory or developmental history. One of the best studied examples concerns the central projections of cricket clavate hairs located on the cerci, a paired sensory appendage at the rear of the cricket. Clavate hair sensory afferents are somatotopically ordered according to circumferential position on the cercus (Murphey et al., 1980). These central projections are also correlated with the birthday of the neuron (Murphey et al., 1980). Examination of clavate hair afferents on supernumerary cerci, which occur at the same position on the cercus but are born later than their counterparts, has shown that these neurons often follow atypical axon trajectories yet project to the CNS in accordance with their location on the supernumerary structure (Murphey et al., 1983b). This indicates that the segregation of clavate hair afferents within the CNS is based on peripheral location, not birth order.

In *Drosophila*, the influence of developmental history on central axonal projections has been addressed. The leg is divided into an anterior and a posterior developmental compartment, based on the expression of *engrailed*. Tactile bristles on the leg form either an anterior projection or a posterior projection, and within each main projection shape also form a somatotopic map that is related to circumferential position around the leg (Murphey et al., 1989b). The choice as to which projection pattern to make is not determined by developmental history as the line that determines axon compartment does not correspond to the point where axon trajectory changes from anterior to posterior. Additionally in *engrailed* mutants, where a transformation of posterior compartment identity towards anterior compartment identity occurs, afferent projections are essentially normal even when accompanied by a change in external appearance (Murphey et al., 1989b). Here too initial axon trajectory does not determine the ordering of sensory afferents, as axons that enter the CNS through the anterior axon bundle can still cross over and produce the alternate projection pattern. In both the cricket and *Drosophila*, peripheral location appears to be encoded during the differentiation of sensory neurons and that positional information determines target region within the CNS.

By creating chimaeric sensory systems in crickets, Walthall and Murphey were able to test the role of positional information in guiding sensory projections. Transplantation of cercal epidermal tissue containing identified sensory neurons from a black cricket to abnormal locations on the host cercus of a tan cricket, enabling donor receptors to be identified, determined that the transplanted neurons arborise in locations consistent with their original position (Walthall and Murphey, 1984). Such heterotypic transplants trigger epidermal cell proliferation along the border of the graft, and the differentiation of new sensory receptors. This new tissue represents intermediate locations between the positional values of the donor and host epidermis (French et al., 1976). Walthall and Murphey showed that the newly produced sensory neurons arborise in locations intermediate between those corresponding to the original site of the donor sensory neurons and their new position (Walthall and Murphey, 1984), providing direct support for the notion that the relative position of the cell body when a sensory neuron differentiates determines central projection area.

Such maps provide a central representation of the external world, preserving information regarding stimulus direction or sound frequency within the CNS, and presenting these data to post-synaptic interneurons and motorneurons in an accessible form. The organisation of sensory maps and the modality-specific segregation of sensory afferents has been shown to be related to the morphology and receptive field of post-synaptic interneurons. In the cercal-to-giant-interneuron system of the cricket, the wind-sensitive hairs that cover the cercus are

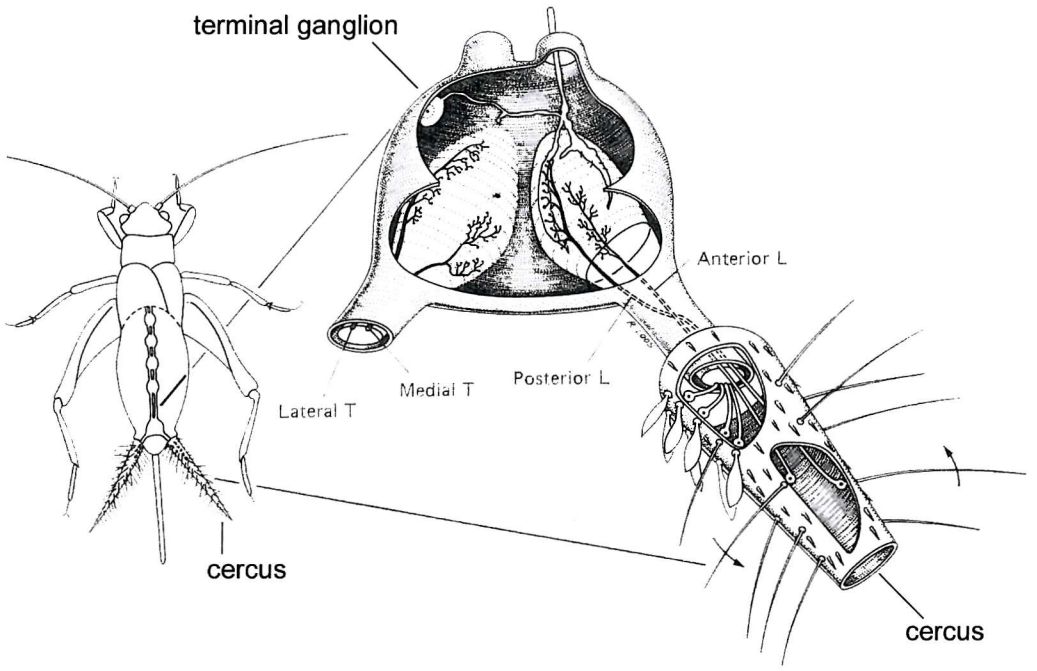


Figure 1.2. The Cricket Cercal-To-Giant Interneuron System. A cut-away view of a cricket is shown on the left, illustrating the location of the CNS, and a detailed view of the cercus and terminal abdominal ganglion is shown on the right. Sensory neurons at different locations on the cercus respond to different wind directions (illustrated by arrows) and project to different regions of the terminal abdominal ganglion. The projection patterns of the four types of sensory afferent, Lateral T, Medial T, Posterior L and Anterior L are depicted. The postsynaptic cells have their dendrites restricted within the target area and are therefore contacted only by sensory neurons that project to that area. This is illustrated for the medial giant interneuron, which can be seen to contact the posterior L afferent but not the anterior L afferent. From Bacon and Murphey (1984).

each responsive to different wind directions. Sensory neurons of similar directional sensitivities are clustered together in strips on the cercus. Each strip projects to a different area of the CNS, such that different regions of the CNS receive sensory afferents coding for different wind directions (Bacon and Murphey, 1984). Each of the giant postsynaptic interneurons has dendrites restricted to portions of neuropil corresponding to the different wind directions and only responds to input from those specific wind directions, demonstrating that the receptive field of these giant interneurons is a function of their morphology and is correlated with the organisation of pre-synaptic sensory afferents (Bacon and Murphey, 1984). This is shown in Figure 1.2.

In the locust the receptive fields of spiking local interneurons overlap to produce a complete map of the hind leg (Burrows and Siegler, 1984), and here too the morphology and receptive field of these spiking local interneurons corresponds with the somatotopic tactile afferent map (Newland, 1991). When specific branches of a cricket interneuron were ablated using laser microsurgery, the interneuron stopped receiving input from the sensory afferents that normally overlap those dendritic branches (Jacobs and Miller, 1985). This showed that changing the morphology of an interneuron resulted in a corresponding change in its receptive field. The significance of anatomical segregation of axonal arbours and interneuron dendrites is further demonstrated by the observation that ectopic bristle afferents on transplanted cerci project to the appropriate region of ventral neuropil and are able to form functional connections in the thorax with the corresponding interneurons whose dendrites are restricted to bristle neuropil (Murphey et al., 1985). Motorneurons also exhibit spatial restrictions in their dendritic arborisation domains, which are related to the position and orientation of the muscles they innervate (Landgraf et al., 1997). These highly ordered projections of sensory afferents and the corresponding anatomical organisation of post-synaptic interneuron and motorneuron dendrites are thought to be an important requirement for the formation of specific synaptic connections. Such segregation physically restricts the set of target neurons available to a given neuron, thereby helping to ensure that sensory neurons meet and form synapses with the appropriate partners.

While the anatomical segregation of sensory afferents clearly influences synaptic connectivity, it does not determine connectivity. The strength of the synaptic input must be adjusted and in the cricket cercal system competition has been shown to regulate the efficacy of synaptic contacts (Shepherd and Murphey, 1986). A small number of cercal sensory neurons have axons that arborise bilaterally within the CNS. When one cercus was experimentally removed, destroying 90% of the afferents on one side, most of the bilateral neuron's synaptic material

was transferred to the experimental side, where competition was greatly decreased (Murphey and Lemere, 1984). This suggests that the neuron is able to detect the level of neighbouring competition and shift its resources accordingly. These changes in the morphology of the bilaterally projecting neuron have been demonstrated to be accompanied by changes in the strength of its synaptic contacts with the medial giant interneurons (Shepherd and Murphey, 1986). Such interactions also influence the probability of synapse formation (Shepherd and Murphey, 1986).

The importance of competitive interactions in shaping the final pattern of synaptic connectivity is further emphasised by the finding that supernumerary sensory neurons compete with the normal sensory neurons for their target interneurons in the cockroach, reducing the efficacy of the existing connection (Bacon and Blagburn, 1992). In both types of competition experiment sensory afferents never invade atypical regions of neuropil (Bacon and Blagburn, 1992; Murphey, 1985). This indicates that while competition with surrounding sensory neurons may be involved in establishing and refining the patterns of synaptic connections, sensory identity determines afferent location in the CNS.

While a vast body of literature exists to describe the functional organisation of sensory axons within the CNS, little is known regarding the genetic mechanisms that determine sensory projection pattern. As sensory neurons make specific patterns of projections according to their position and their sensory modality, the specification of position and sensory identity therefore plays a pivotal role in the formation of synaptic connections. Insights into the mechanisms governing the positioning of sense organs and determining the type of sense organ to be produced have come from the study of neurogenesis in *Drosophila.*

1.2 Neurogenesis in *Drosophila*

1.2.1 Positioning the neural precursor

As there is little migration in the peripheral nervous system, the positioning of each neuronal precursor is vital in generating the pattern of the peripheral nervous system. The activation of the proneural genes, which are required for the formation of neural precursors (Cubas et al., 1991; Romani et al., 1989), in a specific spatial and temporal pattern within the epidermis therefore determines the positions of the neuronal precursors.

The expression of the proneural genes *achaete* and *scute* of the *achaete-scute* complex (*AS-C*) in the small groups of epithelial cells that give rise to neuronal precursors is thought to be governed by positional information supplied by the anterior-posterior and dorso-ventral patterning genes. Mutations in pair-rule genes have been shown to affect the positioning of neuroblasts in the regions of ectoderm that give rise to the CNS, with individual pair-rule genes causing the deletion or fusion of specific *achaete*-expressing cells (Skeath and Carroll, 1992). While this information may directly activate *achaete* and *scute* in the embryo, there is evidence that certain regions of ectoderm that give rise to neuroblasts may require the action of further genes, such as *ventral nervous system condensation defective* (*vnd*), to translate the primary pre-pattern information (Jimenez et al., 1995). In the adult sensory nervous system, the specification of a subset of bristles is dependent on the activity of the genes of the *iroquois* complex. These genes interpret pattern information provided by *decapentaplegic* and *cubitus interruptus* (Gomez-Skarmeta et al., 1996; Gomez-Skarmeta and Modolell, 1996). Both members of the *iroquois* complex encode closely related homeodomain proteins which bind to regulatory regions of the *AS-C* (Gomez-Skarmeta et al., 1996), suggesting that they directly regulate transcription of the *AS-C*. In addition, the zinc-finger protein, Pannier, can act as a negative or positive regulator of *AS-C* transcription, depending on where it is expressed (Ramain et al., 1993).

The specified pattern of sense organs varies depending on segmental identity. If the activity of proneural genes is regulated by the anterior-posterior and dorso-ventral patterning genes, it seems likely that the differences in peripheral nervous system (PNS) pattern between segments may be dependent on the homeotic genes that control segmental identity. Loss-of function mutations in the homeotic genes generally appear to affect PNS development as a by-product of their effect on the epidermis, but there do appear to be some exceptions. *Sex combs reduced* affects the morphology and position of the chordotonal cluster in the prothoracic segment, while loss of *Antennapedia* results in a loss of external sensory neurons in the thoracic segments and the appearance of several ectopic chordotonal neurons (Heuer and Kaufman, 1992). *Ultrabithorax* is required for the formation of all sense organs derived from the anterior compartment in segment A1, and loss of *abdominal-A* activity causes the five lateral chordotonal neurons of abdominal segments to be transformed into the dorsal cluster of three chordotonal neurons present in thoracic segments (Heuer and Kaufman, 1992). These homeotic mutations do not affect the lineages of sense organ precursors or the type of sense organ formed. Ectopic expression of these homeotic genes also results in defects in PNS development, but tends to cause a reduction in sensilla development that cannot be easily explained (Heuer and Kaufman, 1992). As PNS development was found to be more

susceptible to overexpression of homeotic genes at 3-4 hours of development, the period prior to the appearance of precursor cells, this suggests that homeotic genes may participate in regulating the expression of the proneural genes, which begin to be expressed around this time.

Ubiquitous activation of proneural genes results in the formation of additional sense organs (Garcia-Bellido, 1979; Jarman et al., 1993). The positioning of ectopic sense organs is not random however and suggests that some regions of the embryo are refractory to proneural gene activity. Loss of function mutations in the genes *hairy* and *extramacrochaete* (*emc*), on the other hand, causes additional external sense organs to be produced. Both genes show dosage-sensitive interactions with members of the *achaete-scute* complex such that the ectopic sensilla produced in *emc* and *hairy* mutants is partially suppressed in AS-C heterozygotes and enhanced by a duplication of the *AS-C* (Del Prado and Garcia-Bellido, 1984; Ingham et al., 1985b; Ellis et al., 1990). Furthermore, these ectopic sense organs require *AS-C* function for their formation and can appear in regions of the embryo where sensilla are not normally located (Alonso and Garcia-Bellido, 1988; Ellis et al., 1990). This indicates that *emc* and *hairy* function to suppress the formation of sensory organs in aberrant locations by antagonising *achaete* and *scute* in those regions. Acquisition of neuronal competence in clusters of ectodermal cells may therefore require the reduction or absence of *emc* and *hairy* activity, in addition to proneural gene expression. Consistent with this, it has been shown that *emc* expression is low in neurogenic areas (Van Doren et al., 1992).

1.2.2 Singling out the neuronal precursor

Sense organs are derived from single neuronal precursor cells, sense organ precursors (SOPs), which delaminate from the developing ectoderm. Proneural genes begin to be expressed in small patches of epidermal cells (proneural clusters) and endow these cells with the ability to become neural precursors (Cubas et al., 1991; Romani et al., 1989). The expression of these genes is then restricted to a single cell by a process of lateral inhibition and this remaining neuronally competent cell will become the neuronal precursor. This is shown in Figure 1.3.

Sense organ precursors appear to be selected from a smaller group of cells within the proneural cluster that accumulate higher levels of proneural proteins than their neighbours, with the cell eventually destined to become the SOP accumulating the highest levels of all (Cubas et al., 1991; Cubas and Modolell, 1992; Skeath and Carroll, 1991). The selection of a

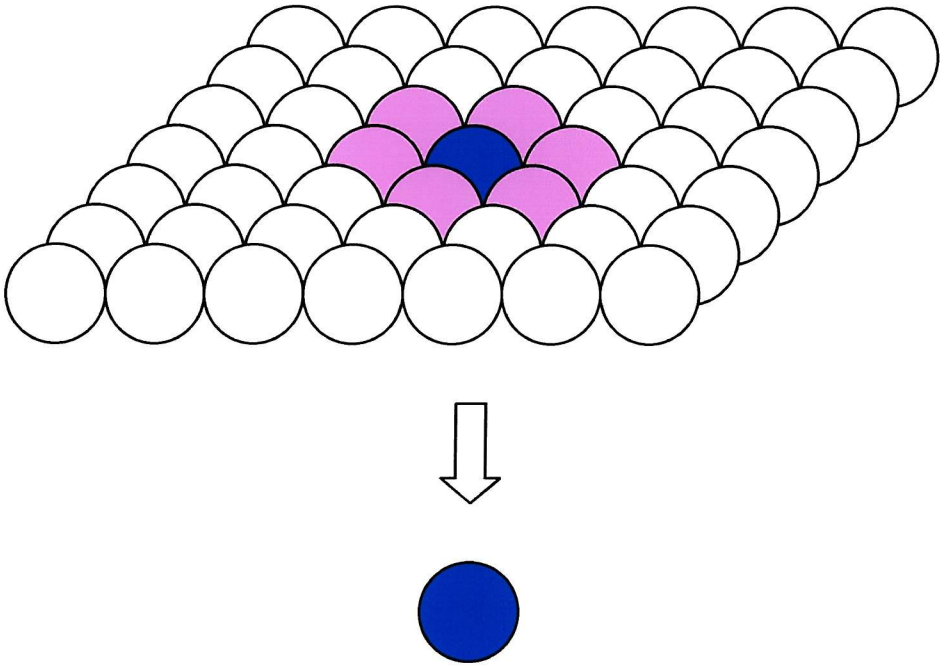


Figure 1.3 Sense organ precursor formation. Proneural genes are activated in small cluster of ectodermal cells and endow these cells with the competence to become SOPs. The SOP is then selected from this group of cells by accumulating a higher level of proneural proteins than the neighbouring cells. Once the SOP has accumulated the highest levels of proneural proteins it will delaminate from the neuroectoderm and initiate neuronal differentiation.

single precursor from each group of neuronally competent cells is achieved through a process of lateral inhibition mediated by Notch-Delta signalling (Artavanis-Tsakonas et al., 1995). Binding of the Notch receptor by its ligand, Delta, induces the expression of the genes of the *Enhancer of split (E(spl))* complex, which function to repress proneural competence in the cells of the cluster receiving the Notch signal (Jennings et al., 1995; Jennings et al., 1994). The *E(spl)* complex encodes seven bHLH transcriptional repressors (Klambt et al., 1989) and these may directly repress expression of the proneural genes.

Two models have been proposed to explain how this type of mechanism can lead to the singling out of a precursor (Artavanis-Tsakonas and Simpson, 1991; Ghysen and Dambly-Chaudiere, 1993; Muskavitch, 1994). Firstly small differences in neural competence between cells could become magnified through competitive inhibition between neighbouring cells (Artavanis-Tsakonas and Simpson, 1991; Ghysen and Dambly-Chaudiere, 1993). *Notch, delta* and the proneural genes would therefore operate via an intercellular negative feedback loop which would exaggerate any small increases or decreases in competence between cells until one cell establishes itself as the only neurally competent cell. Alternatively Notch-Delta signalling could cause all cells to remain in a state of inhibition until one cell, perhaps through the accumulation of proneural proteins, ceases to be inhibited and becomes the precursor (Muskavitch, 1994). Evidence has accumulated for both theories, and it remains possible that both processes occur but represent different stages on the path to selecting a single precursor.

The neuronal precursor always occupies a very similar position in the cluster, however. Prepattern factors have been suggested to predetermine a small subset of the proneural cluster cells to become the SOP (Culi and Modolell, 1998). Several other unidentified factors are also thought to be required for the accumulation of proneural gene expression in the SOP (Culi and Modolell, 1998). One such factor may be Senseless, a novel zinc finger transcription factor required for the proper development of the PNS (Nolo et al., 2000). Loss of function mutations abolish the upregulation and maintenance of proneural expression in the neural precursor, and ectopic expression results in the production of ectopic sense organs. Senseless activity is restricted to the subset of cells that express the highest levels of proneural proteins and is dependent on proneural gene function, suggesting that proneural genes activate *senseless* which in turn is required to activate and maintain proneural gene expression in the SOPs (Nolo et al., 2000). This may be accomplished by direct activation of proneural gene transcription, possibly in conjunction with transcriptional inhibition of the *E(spl)* complex genes (Nolo et al., 2000).

1.2.3 Sense organ formation

1.2.3.1 Proneural genes

Loss of function mutations in proneural genes delete specific subsets of sensory organs, and ectopic expression results in the formation of additional sense organs, indicating these genes are required for the specification of sensory precursors (Garcia-Bellido, 1979; Jarman et al., 1993). A number of proneural genes have been identified and include *achaete* (*ac*), *scute* (*sc*) *lethal-of-scute* (*lsc*) and *asense* (*ase*), which together comprise the *achaete-scute* complex (*AS-C*) (Cabrera et al., 1987; Romani et al., 1987; Alonso and Cabrera, 1988); *amos* (Goulding et al., 2000; Huang et al., 2000); and *atonal* (Jarman et al., 1993). The *AS-C* genes, *atonal, amos, emc* and *hairy* all encode Helix-Loop-Helix (HLH) proteins (Alonso and Cabrera, 1988; Garrell and Modolell, 1990; Jarman et al., 1993; Rushlow et al., 1989; Goulding et al., 2000; Huang et al., 2000) which are expressed in the proneural clusters and sense organ precursors (Cabrera et al., 1987; Romani et al., 1987). The HLH motif is important for protein dimerisation and HLH proteins can generally form both homodimers and heterodimers. Most HLH proteins, including the proneural gene products, also have a conserved basic motif adjacent to the HLH region (b-HLH), which enables the protein to bind DNA with sequence specificity and to function as transcription factors (Cabrera and Alonso, 1991). The AS-C proteins and Atonal bind to DNA as heterodimers with the ubiquitously expressed b-HLH protein Daughterless (Cabrera and Alonso, 1991). Neuronal precursors still form in *daughterless* mutants but cannot progress through cell cycle, indicating that *daughterless* is not required for the specification of neuronal fate but is necessary for the differentiation of the neuronal precursor (Hassan and Vaessin, 1997). EMC lacks the basic DNA-binding region, suggesting EMC may antagonise AS-C function directly by binding to their dimerisation domains and preventing heterodimer formation and inhibiting subsequent DNA-binding (Garrell and Modolell, 1990; Ellis et al., 1990). Indeed, EMC has been shown to be able to heterodimerise with members of the AS-C and prevent DNA-binding in vitro and transcriptional activation in vivo (Cabrera et al., 1994). *Hairy* does encode a b-HLH protein, and is therefore thought to directly repress *ac* transcription (Van Doren et al., 1994).

Different proneural genes specify different subsets of sense organ precursors. In the embryonic sensory nervous system the *achaete* and *scute* genes of the *AS*-C are required for the formation of the external sense organ precursors and a subset of multidendritic precursors (Campuzano and Modolell, 1992). Different members of the *achaete-scute* complex can compensate for each other functionally (Rodriguez et al., 1990; Dominguez and Campuzano,

1993), indicating a high degree of redundancy in the cluster. For the *achaete* and *scute* genes it has been shown that both are initially activated in complementary spatial domains under the control of the individual *cis*-regulatory sequences of each gene, and subsequently each gene activates the other to produce corresponding patterns of expression (Martinez and Modolell, 1991). Other potential targets of AS-C transcriptional activation include the neurogenic genes, such as the *E(spl)* complex (Hinz et al., 1994), and genes required for specifying neural identity and controlling neural differentiation such as *cut* and *pox-neuro* (Blochlinger et al., 1990; Dambly-Chaudiere et al., 1992; Vaessin et al., 1991).

Atonal controls the formation of the chordotonal organ precursors (Jarman et al., 1993). Analysis of *atonal* (*ato*) mutants has shown this gene to be required for chordotonal precursor formation, with the exception of one of the abdominal pentascolopidial organ neurons which, it appears, can be specified by the *AS-C* in the absence of *ato* (Jarman et al., 1993; Jarman et al., 1995). Of the eight chordotonal organs that are present in each hemisegment of the *Drosophila* embryo only five of the neural precursors express *ato* transcripts: these include the neuron formed in *ato* mutant embryos (zur Lage et al., 1997). The remaining three *atonal*-dependent chordotonal precursors are recruited by the *ato*-expressing founder cells via a local signalling mechanism involving the *Drosophila* EGF receptor (DER) pathway (Okabe and Okano, 1997; zur Lage et al., 1997). In AS-C and *atonal* double mutant embryos, a small subset of multidendritic neurons remain (Dambly-Chaudiere and Ghysen, 1987; Jarman et al., 1993). These are specified by *amos*, a recently identified proneural gene related to *atonal* (Goulding et al., 2000; Huang et al., 2000).

While proneural genes are only briefly expressed in the SOP during sense organ differentiation (Cabrera et al., 1987; Romani et al., 1987), the expression of specific proneural genes must play some role in determining the identity of the sense organ produced, as ectopic expression of *achaete-scute* complex genes will cause the formation of ectopic external sense (es) organs but never chordotonal (ch) organs (Rodriguez et al., 1990; Jarman et al., 1993). The mechanisms controlling asymmetric cell division are common to both es and ch lineages, yet each will eventually produce very different sense organs with different roles in the nervous system. It appears that this difference is established in the sense organ precursor. Different proneural genes are required for the specification of es, ch, and some md precursors, and the genes that confer the identity of the sense organ lineage may be targets of proneural transcriptional activation.

1.2.3.2 Identity selector genes

A number of genes have been identified which specify the type of sense organ to be formed. One such gene is *cut*, which is expressed in *AS-C* dependent precursors but not in *ato* dependent precursors (Blochlinger et al., 1990). Loss of *cut* activity causes the neurons and support cells of the es organs to be transformed into those of ch organs. Ubiquitous expression transforms ch organs into es organs but does not cause the formation of ectopic sense organs, indicating *cut* is required for es organ identity but cannot initiate sense organ development (Blochlinger et al., 1991). *BarH1* and *BarH2* encode two functionally redundant homeobox genes that appear to play a role in determining what type of es organ is produced (Higashijima et al., 1992). Alone, loss of function mutations have no effect on development. Deletion of both transforms certain es organs from campaniform-like sensilla to trichoid sensilla and overexpression of either gene causes the complementary change in fate (Higashijima et al., 1992). Expression of *BarH1* and *BarH2* is strongest in the campaniform-like sensilla es precursors and is later found in the glial cells and neurons of these es organs.

The gene *pox-neuro* (*poxn*) has also been shown to be required in the es lineage for the development of the poly-innervated es (p-es) organs and is expressed in a subset of the es precursor cells that give rise to the p-es organs (Dambly-Chaudiere et al., 1992). In addition to being able to transform mono-innervated es (m-es) organs into p-es organs when it is ectopically expressed, *poxn* can also transform ch precursors into es precursors by inducing *cut* expression (Vervoort et al., 1995). One explanation for this interesting result is that both genes are required to specify p-es formation and that direct activation of *cut* by *poxn* ensures that both are always co-expressed in those precursors (Vervoort et al., 1995). The scenario that emerges is that, in the absence of *cut* and *poxn* activity, a sense organ precursor will develop as a ch organ and that, in the absence of *poxn* expression, an es organ will develop as a mono-innervated es organ. Other factors such as *BarH1* and *BarH2* are required to specify the exact type of sensory hair produced by the es organ. One reason the specification of ch organ precursors may require a separate proneural gene, *atonal*, is therefore that *cut* and *poxn* are not targets of *atonal* transcriptional activation, enabling those precursors to develop as ch organs.

A number of genes have therefore been identified which are required for the formation of neural precursors and for sense organ specification. As many of these genes play a role in determining the type of sense organ to be formed, such genes may also govern sensory projection pattern within the CNS.

1.3 Genetic mechanisms governing the formation of an ordered array of sensory projections

Although several of the genes that determine the type of sense organ to be produced have now been identified, few studies have examined the relationship between the specification of sensory modality and the formation of an ordered pattern of central projections. In the *Drosophila* embryo the different types of central projection correlate closely with the proneural gene required for sense organ precursor formation, suggesting that the proneural genes may play an important role in determining sensory identity (Merritt and Whitington, 1995). The expression of proneural genes cannot directly determine central projection pattern however as proneural genes are only expressed in neural precursors and not in the sense organs themselves (Cabrera et al., 1987; Romani et al., 1987). As discussed above, one of the targets of proneural transcriptional activation may be the gene *cut*, which is required for es organ identity and is only expressed in *AS-C* dependent precursors (Blochlinger et al., 1990; Blochlinger et al., 1991). The role of *cut* in specifying terminal arbours has been investigated by examining the projections of es neurons in embryos mutant for *cut*, where the neurons and support cells of the es organs are transformed into those of ch organs (Merritt et al., 1993). The central projections of transformed es neurons are variable: some appear almost wild-type, some resemble those of ch neurons, while others do not conform to either type. This would indicate that while *cut* influences neuronal projections it is not the sole determinant of external sensory neuron identity. In the absence of *cut* some aspects of es neuron identity remain and contribute to the specification of the es central projection pattern. While *cut* clearly does not function as a simple modality control switch for all aspects of neuronal identity, this does not preclude the possibility that the upstream proneural genes could satisfy this function. In the adult however, ectopic expression of *pox-neuro*, required for the specification of poly-innervated (chemosensory) es organs (Dambly-Chaudiere et al., 1992), results not just in the transformation of mechanosensory bristles into chemosensory bristles but also in the underlying neuron establishing a central projection pattern similar to wild type chemosensory neurons (Nottebohm et al., 1992). This transformation results in the formation of a functional chemosensory receptor, suggesting that *pox-neuro* at least is capable of specifying multiple aspects of neuronal identity (Nottebohm et al., 1992).

The role of pre-pattern genes in determining central projection pattern has also been examined for mechanosensory neurons on the *Drosophila* notum (Grillenzoni et al., 1998). Medially located macrochaetes have contralateral branches to their central projections whereas the

arborisations of lateral macrochaetes are restricted to the ipsilateral side of the CNS. The specification of the lateral bristles is dependent on *aracaun* and *caupolican*, two members of the *iroquois* complex, which interpret pattern information provided by *decapentaplegic* and *cubitus interruptus* and are required for the activation of the proneural genes *ac* and *sc* (Gomez-Skarmeta et al., 1996; Gomez-Skarmeta and Modolell, 1996). Loss of function mutations in the *iroquois* complex result in a failure of lateral bristles to form the characteristic lateral projection whilst ectopic expression of either *araucan* and *caupolican* causes medial microchaetes to assume a lateral projection (Grillenzoni et al., 1998). The central projection of the medial macrochaetes however remains unaffected, although it is possible that this may reflect limitations of the experimental set-up. Nevertheless, these results strongly suggest that the genes of the *iroquois* complex control lateral identity for a subset of notal mechanosensory neurons. The effect of the proneural genes *achaete* and *scute* on the formation of lateral and medial projections were also investigated and found, perhaps unsurprisingly given the high degree of redundancy between the *achaete-scute* complex genes (Rodriguez et al., 1990; Domingues and Campuzano, 1993), to have no correlation with lateral or medial identity (Grillenzoni et al., 1998)

As pre-pattern genes activate proneural gene expression in the epidermis, the effect of the *iroquois* complex genes on central projection pattern must again however be indirect. While the specification of position and sensory identity are important determinants of central projection pattern, the mechanisms by which positional information and sensory modality are translated into a precise pattern of central projections remain largely unidentified. A discussion of the molecular basis of axon guidance is of relevance to this question as many of these genes may also be required for the formation of the sensory afferent projection.

1.4 Development of neuronal connections

The functioning of the nervous system is dependent upon intricate and highly ordered networks of synaptic connections. Neurons become organised into functional arrays of neuronal connections with incredible fidelity during development, despite frequently navigating considerable distances to find their appropriate targets. Remarkable advances in our understanding of the cellular and molecular basis of axon guidance have been made in recent years.

1.4.1 Pioneer neurons and pathway formation

The first neuronal pathways are established early in development when the distances to be travelled are often short and the embryonic landscape still relatively simple. These pathways form the major nerve routes and provide an axonal scaffold which later born neurons contact and migrate along to bring them to their target region. This led to the suggestion that the early growing axons that first navigate these pathways function as 'pioneer' neurons (Bate, 1976; Goodman and Bate, 1981; Goodman, 1984; Ho and Goodman, 1982; Keshishian, 1980; Keshishian and Bentley, 1983a; Keshishian and Bentley, 1983b; Keshishian and Bentley, 1983c). The pioneer extends along a series of guidepost cells (Bate, 1976; Ho and Goodman, 1982; Bentley and Keshishian, 1982) which provide essential guidance cues to the navigating growth cone (Bentley and Caudy, 1983). Guidepost cells therefore reduce the task of correctly navigating to an often distant target to crossing the short distances between each intermediate target, distances that can be surmounted through filipodial extension. In *Drosophila*, central pioneer neurons contact and extend along the surface of glia (Jacobs and Goodman, 1989a; Jacobs and Goodman, 1989b). These glia occur in positions which prefigure the major axon tracts in the CNS and are thought to provide guidance cues to the pioneer neurons (Jacobs and Goodman, 1989a; Jacobs and Goodman, 1989b). Consistent with this, mutations that affect the position of longitudinal glia disrupt longitudinal tract formation (Jacobs, 1993) as does their ablation (Hidalgo et al., 1995). The midline cells of the Drosophila central nervous system also represent an important intermediate target in the CNS, providing attractive cues to commissural axons while simultaneously forming a repulsive barrier to ipsilateral axons (Klambt and Goodman, 1991; Tear et al., 1996; Kidd et al., 1998).

While pioneer neurons develop in a axon-free environment, later born neurons are faced with a complex web of possible axon pathways. Follower neurons show remarkable precision in their pathway choices and join (fasciculate with) specific axon bundles (Bastiani et al., 1984; Raper et al., 1983b). This observation gave rise to the "labelled pathway hypothesis", where each tract is uniquely labelled by the expression of cell surface recognition molecules, enabling neurons to identify and selectively fasciculate with the appropriate pathway (Raper et al., 1983a; Raper et al., 1983b; Batsiani et al., 1984; Goodman et al., 1983).

The exact role of pioneer neurons in the correct development of axonal pathways remains contentious however. For example, when axonogenesis of the Ti1 neurons, which pioneer a major nerve (5B1) in the developing grasshopper limb, is blocked follower neurons can establish a normal nerve route. This would suggest that nerve tract formation is not dependent

on the presence of pioneer neurons and that pioneer neurons do not have a unique pathfinding ability (Keshishian and Bentley, 1983b). However, the Ti1 pioneer neurons are required for the passage of more distal neurons across the limb-segment boundary (Klose and Bentley, 1989). By advancing over the segment boundary cells before they have acquired the high affinity for growth cones which halts the extension of later born neurons, pioneer neurons are able to provide a bridge across this obstacle to later differentiating neurons (Klose and Bentley, 1989).

The presence of sensory neurons that persist through metamorphosis in holometabolous insects suggests that these persistent sensory neurons may function in an analogous manner to guide the newly produced adult sensory neurons across the larger and more complex adult landscape (Shepherd and Smith, 1996; Williams and Shepherd, 1999; Tix et al., 1989a; Tix et al., 1989b; Lakes-Harlan et al., 1991). Here too the evidence for a developmental requirement for persistent neurons is largely lacking. Deletion of larval sensory neurons disrupts the growth of adult sensory neurons in the beetle *Tenebrio* (Breidbach, 1990) but in the antennal system of *Manduca sexta* (Oland et al., 1998) and for retinular axons in *Drosophila* (Kunes et al., 1993) the presence of intact larval nerves is not required for the formation of adult pathways. Usui-Ishihara and co-workers have recently demonstrated however that ablation of persistent multidendritic neurons in *Drosophila*, whose dendrites are associated with ingrowing adult bristle axons, disrupts notal bristle axon trajectories (Usui-Ishihara et al., 2000).

In *Drosophila*, the central projections of persistent larval sensory neurons are retained intact throughout metamorphosis (Shepherd and Smith, 1996). This larval pattern of axonal projections prefigures the terminal arborisations of adult sensory afferents (Shepherd and Smith, 1996), suggesting that persistent sensory neurons may guide the assembly of ordered sensory projections in the adult. In support of this theory, Williams and Shepherd have now shown that ablation of the persistent larval sensory neurons disrupt the central projections of adult sensory neurons in a manner consistent with a guidance function for the persistent sensory neurons (Williams and Shepherd, 2002). Central projection regions could therefore be pioneered during embryogenesis, when the CNS is still relatively small.

The idea that pioneer neurons establish the major axon routes early in development and provide guidance cues for later neurons is now firmly established, despite the lack of an absolute requirement for pioneers. Many of the molecules that provide this initial guidance

information to the navigating growth cone and enable subsequent neurons to make guidance choices have now been identified.

1.4.2 The molecular basis of axon guidance

Axon guidance is mediated by the growth cone, a specialised structure located at the tip of growing axons. Each neuron extends an axon which, led by its growth cone, navigates its way through its environment, often traversing long distances to find its correct target. The idea that growth cones are accurately guided towards their final targets by specific chemotactic cues, the chemoaffinity hypothesis, was first proposed by Sperry (1963) to explain the remarkable precision by which retinal axons find their appropriate targets in the amphibian optic tectum. Since then the molecular mechanisms that underlie axon guidance have been the subject of intense study. This work has lead to the discovery of putative recognition molecules that function much like the chemotactic cues of Sperry's original hypothesis. Growth cones are guided towards their appropriate target cells by a mixture of attractive and repulsive cues in the form of secreted and cell surface proteins, encountering both substrates that are permissive for growth and that inhibit axon extension. This provides four main guidance mechanisms: long-range chemoattraction, long-range chemorepulsion, contact chemoattraction and contact chemorepulsion (Tessier-Lavigne and Goodman, 1996) which act in concert to ensure accurate guidance.

Many guidance molecules have now been identified and found to be highly conserved between vertebrates and invertebrates. Rather than being divided into discrete classes correlated with guidance mechanism, closely related molecules can mediate distinct modes of action. The semaphorin family, for example, includes both secreted and transmembrane proteins that function as long-range and short-range chemorepellents, respectively, in a number of different contexts (Kolodkin et al., 1992; Luo et al., 1993; Matthes et al., 1995). Semaphorin III causes dramatic growth cone collapse when encountered by chick sensory axons (Luo et al., 1993), while Semaphorin I functions to stall and then steer approaching pioneer growth cones in the grasshopper (Luo et al., 1993). A distal-to-proximal gradient of secreted Semaphorin II in the grasshopper limb is also required to define the polarity of axon outgrowth (Isbister et al., 1999). Semaphorins are also involved in the regulation of axonal fasciculation. Their expression on surrounding epithelial cells appears to push axons together, tightening fasciculation in the grasshopper (Isbister et al., 1999; Kolodkin, 1996). In *Drosophila* however, semaphorins promote axon defasciculation for CNS axons and

motorneurons, enabling axons to break away from the main nerves at the appropriate point for them to reach their targets (Yu et al., 1998).

Attractive and repulsive signals include the ephrin, netrin, semaphorin and slit protein families (Tessier-Lavigne and Goodman, 1996). Several members act as bifunctional guidance cues, capable of attracting or repelling advancing growth cones. Attractants and repellents are recognised by distinct transmembrane receptors at the *Drosophila* midline. Frazzled is the receptor for the midline chemoattractant, Netrin, (Kolodziej et al., 1996) whilst Slit repulsion is mediated by the Roundabout (Robo) receptor (Kidd et al., 1999; Kidd et al., 1998). The specificity of the growth cone's response to these guidance cues has been shown to reside in the transmembrane receptors, which determine whether a signal is perceived as attractive or repulsive. Chimaeric receptors containing the ectodomain of Robo and the cytoplasmic domain of Frazzled result in axons being attracted to Slit-expressing cells, while proteins consisting of the ectodomain of Frazzled and the cytoplasmic domain of Robo cause axons being repelled by Netrin-expressing cells (Bashaw and Goodman, 1999).

Cell adhesion molecules (CAMs), such as the immunoglobulin (Ig) and cadherin superfamilies, are also instrumental during axon guidance. These molecules are often expressed on subsets of axons and can mediate cell-cell adhesion, providing a molecular basis for the selective affinity that growth cones display for specific neuronal pathways. Perhaps the most well known group of neuronal cell adhesion molecules are the fasciclins in the grasshopper and *Drosophila. Fasciclin II* encodes an immunoglobulin superfamily cell adhesion molecule (IgCAM) (Grenningloh et al., 1991); Fasciclin I defines a new family of cell adhesion molecules, the Fasciclin I protein family (Zinn et al., 1988; Hu et al., 1998); and Fasciclin III is a novel cell adhesion molecule (Snow et al., 1989). The fasciclins are dynamically expressed during development on different subsets of axon pathways at those regions of the axon which fasciculate together (Bastiani et al., 1987; Patel et al., 1987; Harrelson and Goodman, 1988), and act locally to promote fasciculation (Jay and Keshishian, 1990; Lin et al., 1994).

Loss of function mutations in cell adhesion molecules often result in subtle phenotypes (Grenningloh et al., 1991; Lin et al., 1994; Nose et al., 1994), suggesting a high degree of redundancy and functional cooperation in guidance mechanisms. Indeed, double mutants for adhesion/signalling guidance molecules, such as Fasciclin I and the Abelson tyrosine kinase (Elkins et al., 1990) or Neurotactin and the Derailed tyrosine kinase (Speicher et al., 1998) show synergistic axon guidance defects, suggesting the different pathways are functionally

related and that the presence of either one will suffice for accurate guidance. Overexpression often causes more striking defects. In *fasciclin II* mutants, for example, the subset of CNS axons that normally express Fasciclin II fail to fasciculate properly while motorneurons appear unaffected, whereas overexpression results an inability to defasciculate, causing axons to extend beyond their normal targets (Lin et al., 1994; Lin and Goodman, 1994).

The activity of these cell adhesion molecules is thought to be modulated by other proteins. *Beaten path*, for example, encodes a secreted molecule required for motorneurons to defasciculate at the correct point (Fambrough and Goodman, 1996). Dosage-sensitive interactions with both *fasciclin II* and *connectin*, a cell adhesion molecule expressed by motorneurons, suggests that Beat promotes defasciculation by opposing Fasciclin II and Connectin mediated adhesion (Fambrough and Goodman, 1996). Receptor protein tyrosine phosphotases, such as DLAR and DPTP69D, and receptor protein tyrosine kinases, such as Derailed, are also implicated in the regulation of axon fasciculation and defasciculation (Callahan et al., 1995; Desai et al., 1996).

1.4.3. Growth Cone Extension

The growth cone therefore functions as a sophisticated signal transduction device that is able to respond to both attractive and repulsive cues in its extracellular environment and re-orient its growth accordingly. The structure of the growth cone, which consists of a number of filopodia, lamellipodia and branches extending in different directions enables the growth cone to explore its surrounding environment. When one of the filopodia comes into contact with target cells or a high affinity substrate, it enlarges and sends out secondary processes (Bentley and O'Connor, 1994). The filopodium is stabilised through the local consolidation of microtubules, resulting in that process becoming the direction of future axon extension (Mitchison and Kirschner, 1988). F-actin accumulates differentially in filopodia that are making high affinity contacts (O'Connor and Bentley, 1993) and is depleted in growth cones that have been exposed to repulsive factors (Fan et al., 1993). Attractive and repulsive cues may therefore regulate growth cone steering by regulating local concentrations of F-actin.

Rho family small GTPases play an important role in regulating the actin cytoskeleton (Hall, 1994) and are strong candidates for forming part of the signalling mechanisms that transduce guidance information to the actin cytosketelon. Functional evidence for a role in growth cone guidance comes from *Drosophila*, where expression of constituitively active versions of the Rho family GTPases Drac1 (hereafter referred to as Rac) and CDC42 arrests axon outgrowth

(Luo et al., 1994; Kaufmann et al., 1998) while expression of a dominant negative form of Rac cause motorneurons to bypass their normal targets (Kaufmann et al., 1998). Extracellular guidance signals are thought to be transmitted to the GTPases through the action of guanidine nucleotide exchange factors (GEFs), which activate the small GTPases by stimulating the exchange of GDP for GTP (Luo et al., 1997). The GEFs are large multidomain proteins that are believed to associate with complexes that bind the cytoplasmic domains of cell surface molecules, thus providing the necessary link between guidance cues and GTPase activation. The recent identification of the *Drosophila* GEF Trio provides evidence of a role for GEFs in the regulation of axon guidance. Mutations in *trio* display axon pathway defects and show dosage-sensitive interactions with *rac*, suggesting that Trio activates Rac to regulate axon outgrowth and guidance (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000).

The effects of Rac activation on actin dynamics are mediated in part via the serine/ threonine kinase p21 activated kinase (Pak) (Dickson, 2001). In order to alter the direction of axon extension however, such molecules must be activated in spatially restricted domains within the growth cone. Activation of Pak depends upon the binding of the GTP-bound active form of Rac or CDC42 and on the binding of the SH2-SH3 adaptor protein Dreadlocks (Dock) (Hing et al., 1999). SH2 and SH3 domain adaptor proteins such as Dock can recruit proteins into complexes and also bind receptor tyrosine kinases, providing another possible pathway linking axon guidance receptors to the cytoskeleton. Mutations in *dock* show similar defects in photoreceptor axon guidance to *pak*, which can be rescued by tethering Pak to the membrane, suggesting Dock's main function in axon guidance is to recruit Pak to the membrane (Hing et al., 1999). *Trio*, *rac*, *pak* and *dock* all show dosage sensitive interactions in photoreceptor axon guidance indicating they all act in a common pathway (Newsome et al., 2000). Extracellular guidance cues could therefore locally activate Pak via Trio and Dock to direct the course of growth cone extension.

A clear picture is beginning to emerge of how guidance information is transmitted and received at a molecular level and of the signalling pathways that transduce guidance information to the actin cytoskeleton to effect changes in the direction of axon growth. Similar mechanisms are likely to guide the growth of sensory axons. Such genes provide a means by which cell type information could potentially be translated into a specific pattern of central projections and are potential downstream targets of the genetic mechanisms that specify sensory identity.

1.5 The *Drosophila* embryo provides a model for studying the mechanisms that establish an ordered pattern of central projections.

Larger insects such as crickets and locusts have proven invaluable for analysing the functional organisation of sensory systems. However in order to examine the molecular basis of such organisation one must turn to a more genetically amenable insect. The fruitfly *Drosophila melanogaster* is an ideal organism for genetic analysis and, as in larger insects, sensory neurons make specific axonal projections according to their position and modality (Murphey et al., 1989a; Merritt and Murphey, 1992; Merritt and Whitingdon, 1995). The extremely well characterised genetics of *Drosophila*, augmented by the recent completion of the genome sequence, combined with the abundance of experimental tools available for manipulating gene expression and visualising single cells provide a powerful system in which to analyse the genetic mechanisms underlying the central organisation of sensory axons. The embryonic nervous system in particular provides a simple model for examining nervous system development, and has the additional advantage of being able to examine lethal mutations affecting nervous system development in detail.

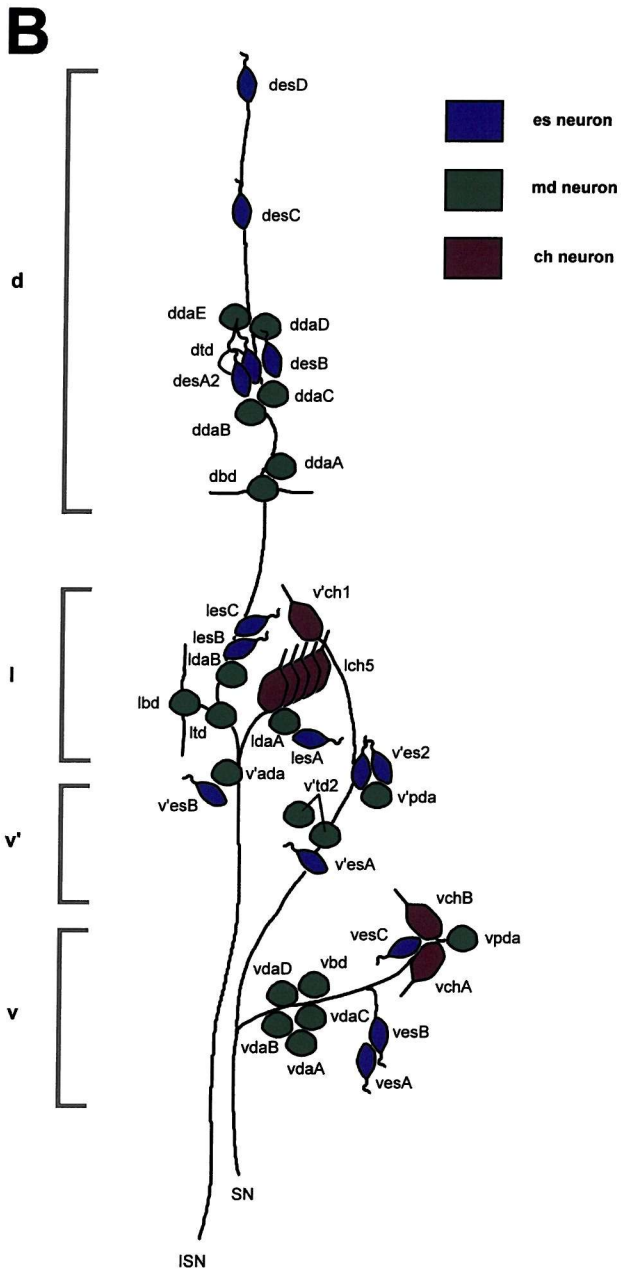
1.5.1 Sensory neurons of the embryonic nervous system and the larval receptors they innervate

The embryonic sensory nervous system comprises a variety of types of sensory structures arranged in a highly ordered and segmentally repeated fashion. There is some variation in pattern, depending on segmental identity, but each segment contains a fixed number of neurons, each of which can be individually identified based on its position and morphology (Dambly-Chaudiere and Ghysen, 1986; Ghysen et al., 1986; Bodmer et al., 1989). The embryonic nervous system is shown in Figure 1.4.

The first seven abdominal hemisegments consist of a completely invariant pattern of 45 neurons, and this basic plan is only slightly modified in the thoracic and terminal abdominal segments. Two main types of sensory neurons are present. The type I neurons present in es and ch organs are bipolar and have a single dendrite, the distal end of which is a modified cilium. Accessory cells derived from modified epidermal cells form the non-neuronal part of the sense organ and produce the stimulus receiving cuticular apparatus. Type II neurons are multipolar and possess several dendrites lacking ciliated structures. These neurons, more commonly known as multidendritic (md) neurons, are generally not associated with support cells so do not form part of a sense organ. The different types of sense organ present and the

Figure 1.4. The Drosophila embryonic sensory nervous system. A. Lateral view of a late stage Drosophila embryo labelled with an antibody against the Futsch protein, which reveals all sensory neurons. Anterior is to the left and dorsal is up. **B.** The distribution of sensory neurons in an abdominal segment, illustrating the different types of sense organ present and the clustering of cell bodies into dorsal, lateral, ventral and ventro-lateral (ventral prime) groups. The abdominal segment shown is representative of A1-A7. es - external sensory neuron, md - multidendritic neuron, ch - chordotonal neuron, v - ventral, v' - ventral prime, l - lateral, d - dorsal, ISN - intersegmental nerve, SN - segmental nerve.


A


B
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md neurons are involved in the reception of different modes of sensory information. These include tactile, chemosensory, proprioceptive, and nocioceptive perception (Bodmer and Jan, 1987; Tracey and Benzer Abstract 56 for the 2001 Annual Drosophila Research Conference, Washington D.C., USA).

The majority of es organs present in the embryonic sensory nervous system are monoinnervated, with the exception of one ventral and one dorsal polyinnervated external sense organ (Hartenstein, 1988). The es neurons innervate the external sensilla of the larvae. Three types of external sense organ are present in the abdominal segments (Dambly-Chaudiere and Ghysen, 1986). Campaniform sensilla are the most numerous. These are circular papillae which are normally surrounded by a ruffled zone, although occasionally occur buried in narrow slits, and are strain receptors. The abdominal papillae p6 is unique in that it is innervated by two neurons, a highly unusual feature for campaniform sensilla, although in external appearance p6 seems identical to all other papillae. Trichoid sensilla are monoinnervated socketed hairs that function as touch receptors. There is also a single poly-innervated dorsal hair. The polyinnervated es organs are chemoreceptors (Dambly-Chaudiere et al., 1992). Larvae also possess sunken basiconic sensilla, which have been described as combined thermo- and hygroreceptors in other insects (Altner et al., 1981).

External sense organs (Figure 1.5 A, C) contain three accessory cells: the thecogen cell, which secretes an extracellular sheath around the tip of the dendrite(s), the tricogen cell which produces the sensory process such as a hair or bristle, and the tormogen cell which forms a circular socket or pit around the base of the sensory process (Hartenstein, 1988). Chordotonal organs (Figure 1.5 B, D) contain between one and five scolopidia, each of which is innervated by a single neuron. A scolopale cell, the thecogen cell homologue, encloses the dendrite and produces an extracellular sheath around its tip (Hartenstein, 1988). The scolopale is connected to a cap cell, which attaches the scolopidium to the epidermis and might be homologous to either the tormogen or the tricogen. A ligament cell secures the opposite end of the scolopidium. The neuron also produces a process of its own which inserts into the epidermis and may provide additional anchorage.

Multidendritic neurons can be divided into three different subclasses: md-da neurons which make abundant dendritic arborisations under the epidermis, md-bd neurons which have bipolar dendrites and md-td neurons, the dendrites of which are associated with tracheal branches (Altner et al., 1981). The md neurons have recently been shown to be nocioceptors (Tracey and Benzer Abstract 56 for the 2001 Annual Drosophila Research Conference,

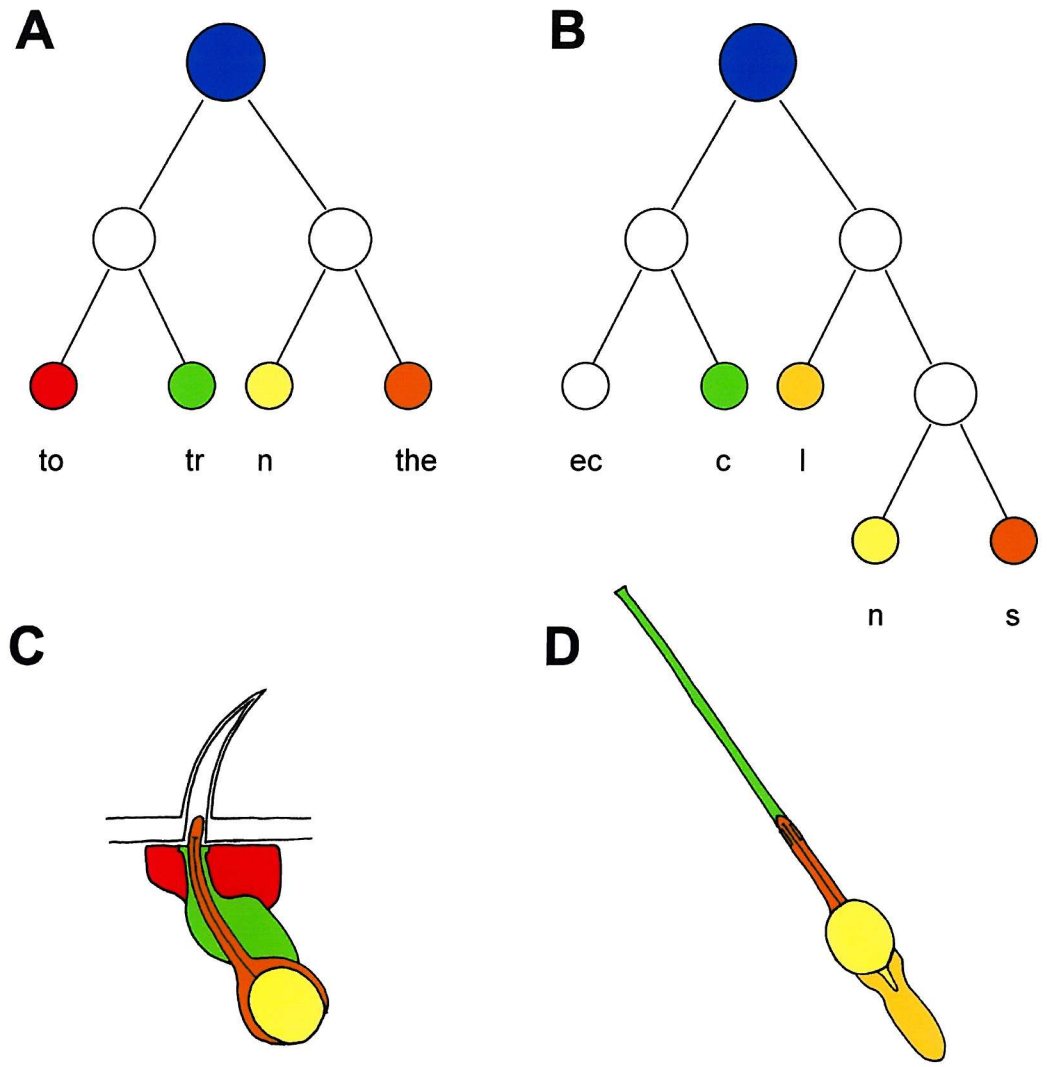


Figure 1.5. Sense Organs and Their Lineages.

A. External sense organ lineage. The SOP (blue) divides to produce two second order precursors. One SOP daughter cell gives rise to the tormogen and trichogen while the other produces the thecogen and the neuron. **B.** Chordotonal organ lineage. The SOP divides to produce two second order precursors. One produces the cap cell and an ectodermal cell. The other gives rise to the ligament cell and another precursor cell, which divides to produce the neuron and the scolopale. The chordotonal lineage is the revised lineage from Brewster and Bodmer, 1995. The ectodermal cell may correspond to an ectodermal attachment cell that has been described as an anchor point for chordotonal organs. **C.** A mechanosensory bristle. **D.** A scolopidium. In both C and D each cell is coloured to indicate the cell it corresponds to in the sense organ lineage. to - tormogen, tr - trichogen, n- neuron, the - thecogen, ec - ectodermal cell, c - cap cell, l - ligament cell, s - scolopale.

Washington D.C., USA) and this function may be mediated by the md-da neurons alone, with members of different subclasses being specialised for different functions.

As there is little migration in the peripheral nervous system and each sensory structure is formed from a single sense organ precursor (Bodmer et al., 1989), the positioning of each neuronal precursor is vital in generating the pattern of the peripheral nervous system (see previous section). Despite the differences between epidermal sense organs and the chordotonal organs, sense organ formation follows a fairly uniform developmental path. Once the pattern of sense organ precursors has been established, each individual precursor will then undergo two rounds of cell division to produce all the cell types necessary for the formation of each particular sense organ (Bodmer et al., 1989; Brewster and Bodmer, 1995). This is shown in Figure 1.5.

Lineage analysis has revealed that md neurons appear to have three different origins. Some md neurons seem to derive from the same precursor that generates certain es lineages, while some appear to be derived from ch precursors. Other md neurons appear to have discrete precursors, as do the majority of es/ch precursors (Brewster and Bodmer, 1995). Loss of *notch*, a gene that plays a role in controlling asymmetric cell divisions in the sense organ lineage, results in an overproduction of md neurons at the expense of es neurons (Vervoort et al., 1997). It has therefore been suggested that some of these 'solo' es and 'solo' md precursors may in fact arise from the earlier division of a single protoprecursor to produce an es precursor and an md precursor. In addition, es precursors predominantly differentiate as md neurons in *string* and *pebble* mutant embryos where cell division is blocked (Vervoort et al., 1997), indicating that a decision concerning which type of neuron to produce in these lineages precedes the choice between the IIa and IIb cell fate in the es precursor.

1.5.2 Central projections of embryonic sensory neurons

Sensory neurons have also been shown to make specific patterns of central projections in the *Drosophila* embryo according to their location and modality (Merritt and Whitington, 1995). Es neurons, ch neurons and md-da neurons all terminate within discrete regions of neuropil, with the remaining multidendritic neurons forming a heterogeneous group. A summary of the projection regions of typical neurons in each group is shown in Figure 1.6. The es neurons are organised somatotopically within the CNS, consistent with their tactile function. Ventral es neurons project posteriorly, and lateral and dorsal neurons project more anteriorly. In addition ventral and dorsal es neurons which are positioned near the dorsal and ventral midlines often

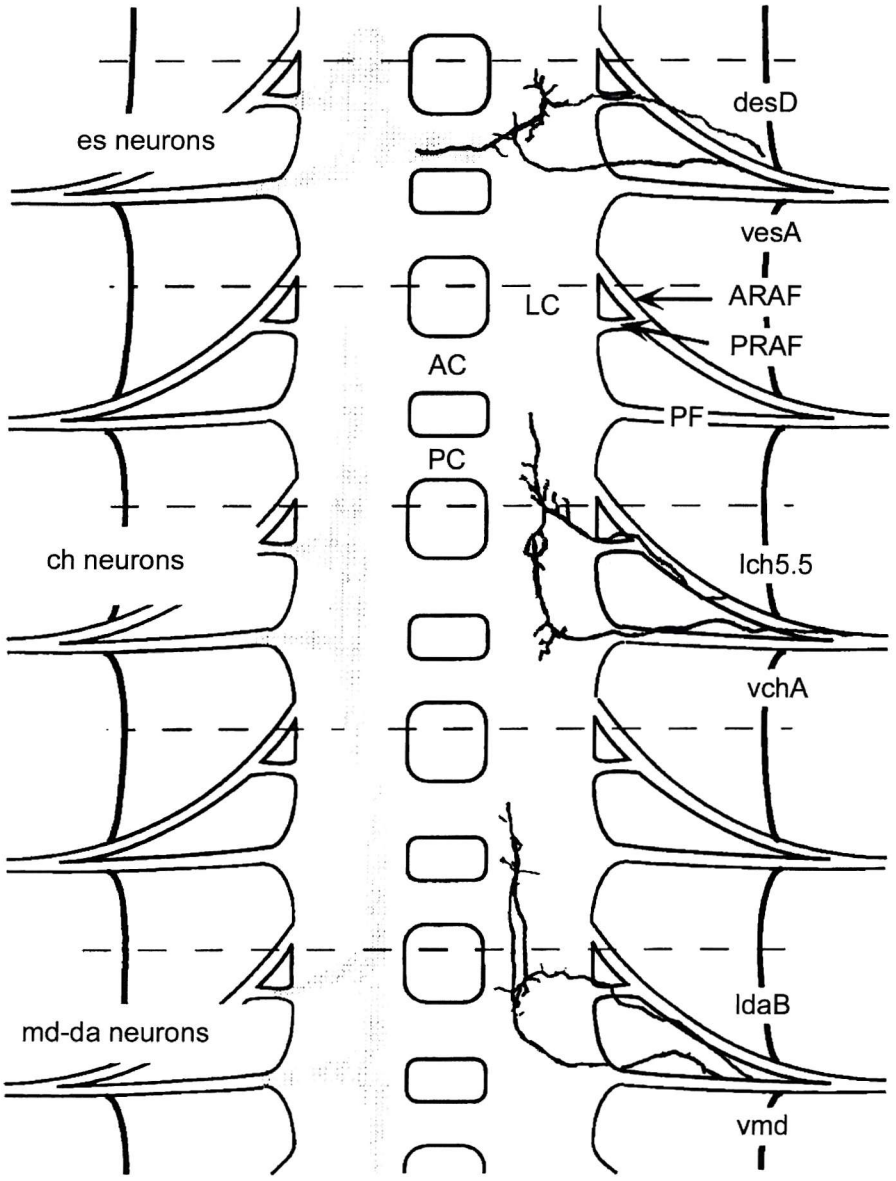


Figure 1.6 Summary of embryonic central projection regions. The stippled area on the left indicates the region occupied by the axons of each group of neurons. On the right, two individual axons are shown, indicating the path taken by axons that enter the CNS through different nerve routes. The ch and md axons occupy discrete longitudinal fascicles within the neuropil. The es neurons are generally more highly branched and are not restricted to longitudinal tracts. Dotted lines indicate segment boundaries. Anterior is up. LC - longitudinal connective, AC - anterior commissure, PC - posterior commissure, ARAF - anterior root of the anterior fascicle, PRAF - posterior root of the anterior fascicle, PF - posterior fascicle. Figure reproduced from Merritt and Whitingdon (1995)

cross the midline within the CNS whilst lateral neurons lack these medial processes. The central arborisations of the es neurons are independent of their point of entry into the CNS; all ventral and v' neurons arrive at the CNS via the posterior fascicle and all lateral and dorsal neuron follow the anterior fascicle yet form graduated somatotopic projections. Ch neurons project to a discrete longitudinal connective within the CNS and have long rostrocaudal processes that do not exhibit obvious somatotopy.

Md-da neurons project to a common sector of the longitudinal connective, and again have long rostrocaudal processes, do not cross the midline and are not visibly somatotopically organised. The lateral tracheal neuron (ltd) was found to share this projection pattern with the majority of md-da neurons, with two notable exceptions: vpda and one dorsal cluster md-da, both of which have a large dorsal component to their axonal projection absent from the characteristic md-da projection pattern. These two neurons and the remaining multidendritic neurons form a fourth heterogeneous population of axon projections. The v'td2 tracheal dendritic neurons extend anteriorly in a lateral fascicle until they reach the metathoracic neuromere where they shift to a dorso-medial fascicle, the dorsal bipolar neuron (dbd) projects dorsally within the CNS, while the ventral bipolar neuron (vbd) runs along the md fascicle before projecting dorsally. The axonal morphology of the lateral bipolar neuron (lbd) has not been described within the CNS.

1.6 This thesis: the identification of genes required for the assembly of sensory arrays in the *Drosophila* embryo.

The *Drosophila* embryonic sensory nervous system provides an ideal system in which to examine the genetic mechanisms that establish an ordered pattern of central projections. While several of the genes that control what type of sensory neuron is formed have already been identified and in some cases their effects of central projection pattern have begun to be examined (Merritt et al., 1993; Grillenzoni et al., 1998; Nottebohm et al., 1992), little is known about how positional information is specified in the sensory nervous system. However, the genes that establish positional values within the developing embryo have been extensively studied (Coulter and Wieschaus, 1986; Dinardo et al., 1988; Fjose et al., 1985; Howard and Ingham, 1986; Ingham et al., 1985a; Jurgens et al., 1984; Nüsslein-Volhard et al., 1984; Siegfried et al., 1994; Wieschaus et al., 1984). Given that signalling pathways are often re-utilised in diverse developmental contexts, such genes may also provide positional information in the sensory nervous system. The aim of this work is therefore to firstly identify candidate genes that could specify positional information in sensory neurons, and secondly to analyse

their contribution to the formation of an ordered pattern of central projections through the development of methods for targeting gene expression to single cells.

Chapter 2. Antibody Screen of the Embryonic Sensory Nervous System

2.1 Aim

An analysis of the patterns of gene activity within the embryonic peripheral nervous system was undertaken with the aim of creating a map of gene expression within each sensory neuron. This information can then be used to identify candidate genes that may shape the projection pattern of sensory neurons within the central nervous system.

2.2 Introduction

Sensory neurons segregate within the CNS according to their sensory modality and position in the periphery (Pflüger et al., 1988; Merritt and Murphey, 1992; Bacon and Murphey, 1984; Ghysen, 1980; Murphey et al., 1989a; Murphey et al., 1989b; Newland, 1991; Merritt and Whitingdon, 1995). The genetic specification of position and sensory modality therefore ultimately determines where a sensory neuron will terminate in the central nervous system, and plays a pivotal role in the development of precise patterns of neural connectivity. Despite their importance, little is known regarding how sensory modality and positional values are first specified in the sensory nervous system, and subsequently translated into a precise pattern of central projections. Several of the genes that specify what type of sense organ is to be formed have now been identified (Blochlinger et al., 1990; Dambly-Chaudiere et al., 1992) yet the mechanisms that govern positional identity remain largely unknown. However, the genes that determine the embryonic axes have been extensively studied in *Drosophila* (Coulter and Wieschaus, 1986; Dinardo et al., 1988; Fjose et al., 1985; Howard and Ingham, 1986; Ingham et al., 1985a; Jurgens et al., 1984; Nüsslein-Volhard et al., 1984; Siegfried et al., 1994; Wieschaus et al., 1984). These genes establish positional values within the embryo and subdivide the embryo into increasingly defined regions, culminating in the specification of anterior-posterior polarity within the 14 embryonic parasegments (reviewed in Hoch and Jäckle, 1993; St Johnston and Nüsslein-Volhard, 1992). As the re-use of the same genetic pathways in different developmental contexts has been an emerging theme of developmental biology, could these same genes be providing positional information to each sensory neuron, thereby contributing to its final pattern of central projections?

The two axes of the embryo are determined by independent dorso-ventral and anterior-posterior systems. The anterior-posterior axis is defined by three different classes of zygotic genes: gap genes, pair-rule genes and the segment polarity genes. These genes, collectively

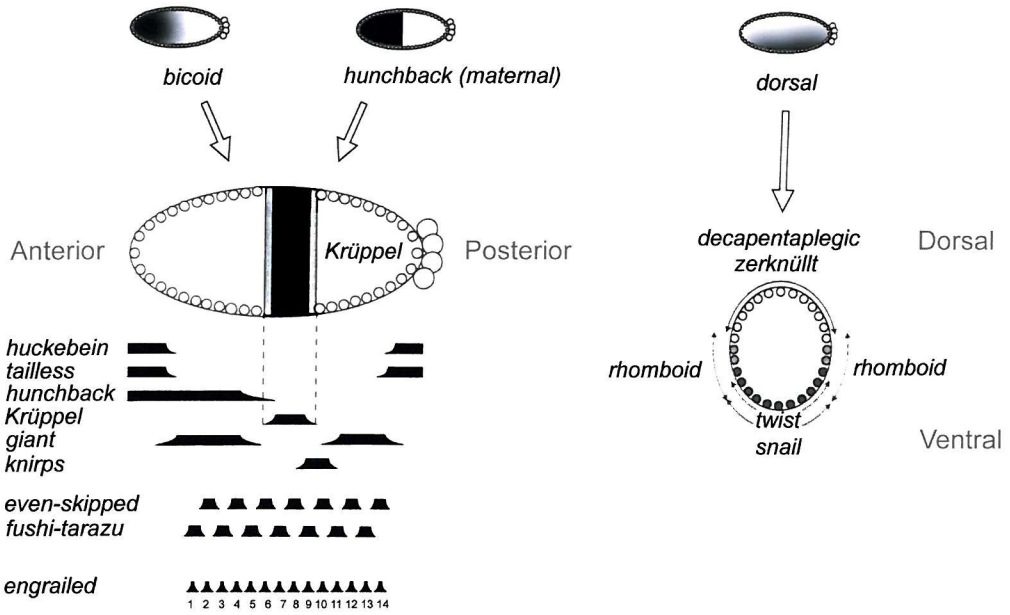


Figure 2. 1 Segmentation and dorso-ventral gene systems that pattern the embryo. The anterior and posterior systems act through gradients of the maternally derived morphogenetic proteins bicoid and hunchback. These locally activate transcription of the zygotic gap gene transcription factors in broad stripes, which form a series of overlapping gradients, which activate the pair-rule genes in seven stripes. The expression of the pair-rule genes in alternate stripes initiates the expression of the segment-polarity genes to produce the segmental pattern. A graded distribution of dorsal in the nuclei along the dorso-ventral (DV) axis causes the zygotic target genes snail, twist, zerknüllt, and decapentaplegic, to be expressed at distinct locations in response to different concentrations of dorsal. The different domains of gene expression along the DV axis are required for several tissues (e.g. amnioserosa, dorsal ectoderm, ventral ectoderm, mesoderm). Adapted from Hoch and Jäckle (1993).

known as segmentation genes, are responsible for the organisation of the embryo into 14 segments. Each class of genes is activated sequentially during development and interact in a regulatory hierarchy whereby each class is transcribed in response to the products of the previous class. The initial expression of gap genes in broad domains along the anterior-posterior axis ensures that the pair rule genes are transcribed in the required pattern of seven stripes. The stripes of pair-rule gene expression determine the positions of the embryonic parasegments, with alternating stripes of pair-rule gene expression specifying the odd and even segments, and activate the expression of the segment polarity genes. Both gap genes and pair-rule genes, which code for transcription factors, are expressed prior to cellularisation and can activate target gene expression directly but the segment-polarity genes are expressed during and after cellularisation and thus many members encode cell-signalling and signal transduction proteins. A second developmental pathway elaborates the dorso-ventral axis. A gradient of Dorsal protein is established by the opposing actions of the dorsal group genes and *cactus*. Dorsal activity regulates the expression of the genes responsible for differentiation along the dorso-ventral axis, with different target genes being activated or repressed in response to different threshold concentrations of Dorsal along the Dorsal gradient (Huang et al., 1997). Both pathways are summarised in Figure 2.1.

It is widely believed that the patterning genes that are active early in embryonic development control the positioning of neuroblasts (Skeath and Carroll, 1992; Skeath et al., 1992). There are therefore two possible mechanisms by which these patterning genes could provide positional information within the developing peripheral nervous system. Firstly, if patterning genes direct the positioning of the sense organ precursors then perhaps positional information is a consequence of the developmental history of a particular sense organ and reflects which area of neuroectoderm the SOP has arisen from. While the SOP is singled out from the proneural cluster by a process of lateral inhibition, the completely invariant position of the SOP has lead to the suggestion that perhaps only 3-4 cells of the proneural cluster are in fact capable of forming the SOP (Culi and Modolell, 1998). The existence of pre-pattern factors has been postulated to predetermine a small subset of proneural cluster cells to become the SOP (Culi and Modolell, 1998) Perhaps the juxtaposition of different positional signalling pathways delineates those cells capable of adopting the SOP fate by activating additional genes within a small region of the proneural cluster and these genes vary depending on where the SOP-competent cells lie within the neuroectoderm, thereby indirectly providing a distinct positional identity to each SOP.

Secondly, these genes may be reactivated later in development in specific sensory neurons and, by their expression, determine positional aspects of neuronal identity and influence axon trajectory within the CNS. Axon guidance decisions depend on the differential expression of cell surface receptors and ligands on specific neurons. As the growth cone encounters different guidance cues, these molecules determine the ability of the neuron to respond to a given cue, and whether that cue is perceived as permissive, attractive, or repulsive. Different central projection patterns therefore probably result from the differential expression of axon guidance molecules by individual sensory neurons. As many segmentation genes are transcription factors, they could directly activate or repress target genes, thereby conferring a distinct expression profile upon that neuron. Target genes potentially include axon guidance molecules, and could provide the long sought link between specification of cell type and axon guidance.

While segmentation genes could theoretically specify positional values in the sensory nervous system, in order to be real candidates for providing positional information it must be determined whether these genes are even expressed during sensory neuron development. The first aim of this project was therefore to establish a clear picture of the patterns of gene expression within the embryonic sensory nervous system. A similar approach has previously been taken by Chris Doe and colleagues, who identified molecular markers for different embryonic neuroblasts to produce a map of these neuroblasts - the hyper-neuroblast map - where each neuroblast could be uniquely identified (Doe, 1992; Broadus et al., 1995). This map has proved invaluable for investigating the mechanisms that specify individual neuroblast fates and determine the different fates of ganglion mother cells (GMCs) within a lineage (McDonald et al., 1998; Weiss et al., 1998; McDonald and Doe, 1997).

I used antibodies or, where antibodies were not available, enhancer-trap lines to examine patterns of gene expression within the sensory nervous system, which enabled me to produce a map of gene expression throughout sensory nervous system development. By creating a map of gene activity within sensory neurons, genes can be identified that are expressed in subsets of sense organs and are therefore candidates for providing positional information to sensory neurons. The role of these genes can then be tested experimentally through ectopic expression and loss of function analysis. Such a map will also enable each neuron to be identified not only by its position and morphology but also by the combination of genes that it expresses. These genes can therefore also be used as markers of cell fate within the sensory nervous system.

2.3 Materials and methods

2.3.1 Fly Stocks

Wild-type Oregon R (OreR) flies were used for antibody screening. The two huckebein-lacZ strains were obtained from Chris Doe (Univeristy of Oregon, USA). The A37 P[lacZ] line was obtained from Alain Ghysen (University of Montpellier, France). Several different GAL4 lines were used: Dll-GAL4; Ptc-GAL4; Dpp-GAL4; hh-GAL4; hairy-GAL4; Ap-GAL4; Dsh-GAL4. All these P[GAL4] lines were obtained from the Bloomington stock centre (Indiana, USA). All flystocks were maintained on standard *Drosophila* media (Appendix A) at room temperature.

2.3.2 Preparation of embryos for Antibody Screening

Embryos were collected overnight on apple-juice agar plates (Appendix A), de-chorionated in bleach and fixed for 20 minutes in a 1:1 mixture of Heptane: 4% paraformaldehyde (Appendix A). After removal of the fixative layer, an equal volume of Methanol was added and the embryos were shaken vigorously for 20 seconds to remove the vitteline membrane*. The embryos were washed in Methanol, and then rehydrated in a series of stepwise washes in 70% Methanol/Phosphate Buffered Saline (PBS; Oxoid, UK) containing 0.01% Tween (Sigma, UK) (PBS-Tw), 50% Methanol/PBS-Tw and then washed three times in PBS-Tw. It is possible to freeze and store the embryos at -20^{0}C while they are in Methanol. Frozen embryos are rehydrated in a series of Methanol/PBS-Tw washes and after washing several times in PBS-Tw are ready for use.

* Some antibodies are sensitive to Methanol devittelinisation and therefore have to be devittelinised by hand. Embryos were collected and fixed as above. The fixative layer was replaced by Heptane and the embryos transferred to an egg basket and left to air dry until all traces of Heptane had evaporated. The embryos were then transferred to a 35mm petri dish lined with double-sided sellotape and covered with 4% paraformaldehyde. Those embryos that adhered to the tape could then be nudged out of their vitteline membranes using a stainless steel minutien pin in a pin holder. The embryos were then transferred to PBS-Tw and processed as normal.

2.3.3 Antibody Screening

All washes and incubations were performed in 1 ml PBS-Tw.

Embryos were incubated for 30 minutes in 2.5% Normal Horse Serum (NHS; Vector, UK) (25 µl in 1 ml PBS-Tw), or 2.5% Normal Goat Serum (NHS; Vector, UK), depending on which serum was an appropriate blocking agent for the secondary antibody being used. O.5 ml was replaced with PBS-Tw to give a final concentration of 1.25% NGS before the addition of the appropriate dilution of primary antibody. Primary antibody dilutions are given in Appendix B. For all primary antibodies, embryos were incubated overnight at 4^0C. Embryos were then washed 3 x 20 minutes and 3 x 1 minute in PBS-Tw.

2.3.4 Fluorescent secondary antibodies

Fluorescent secondary antibodies used were: Cy2 anti-mouse (Jackson, UK) at a dilution of 1:200, Cy3 anti-mouse (Jackson, UK) at a dilution of 1:200, Cy2 anti-rabbit at a dilution of 1:200, Alexa 546 anti-guinea-pig (Cambridge Biosciences) at a dilution of 1:1000, and TRITC anti-rat (Amersham) at a dilution of 1:200. Secondary antibody incubations were performed overnight at 4^0C. Embryos were washed 3 x 20 minutes and 3 x 1 minute in PBS-Tw. For double staining of embryos with different fluorescent secondary antibodies, embryos were processed for both antibodies simultaneously. For double labelling of embryos with a fluorescent secondary antibody and an FITC-conjugated anti-HRP primary antibody (Jackson, UK) the anti-HRP antibody and the fluorescent secondary antibody were both added after primary antibody processing.

2.3.5 Biotinylated secondary antibodies

Biotinylated secondary antibodies used were : anti-mouse (Vector, UK), anti-rat (Vector, UK), anti-rabbit (Vector, UK), and anti-guinea-pig (Vector, UK), all used at a dilution of 1:200. Secondary antibody incubations were performed for 3 hours at room temperature. A Vectastain ABC kit (Vector, UK) was used for the detection of biotinylated secondary antibodies. 10 µl of each of the A and B reagents were mixed in 1 ml PBS-Tw for 30 minutes, and the embryos were incubated in this preformed solution for 30-60 minutes. After 6 x 1 minute washes in PBS-Tw, embryos were transferred to 1 ml PBS-Tw + 30 µl Diaminobenzidine (DAB; Sigma, UK) (final concentration 0.3mg/ml). The reaction was developed by reacting the embryos with 0.003% Hydrogen Peroxide (H_2O_2 ; Sigma, UK).

Embryos were washed 3 x 1 minute in PBS-Tw to stop the reaction. For double staining of embryos with two different antibodies, 60 µl of 1% NiCl solution can be added to the DAB solution to produce a black immunoprecipitation reaction for one antibody, which can be distinguished from the brown immunoprecipitation reaction produced with DAB alone. After completion of the first antibody reaction, embryos are washed 6 x 1 minute in PBS-Tw, before repeating the antibody protocol for the second antibody reaction. The black immunoprecipitation reaction should be carried out first for the antibody that stains the more internal cellular compartment, otherwise the black reaction product will obscure the brown reaction product.

2.3.6 Mounting embryos for examination

All embryos were cleared first in 50% glycerol for 30mins and then in 70% glycerol for 1 hour. Embryos were mounted in 70% glycerol. The embryos were mounted between two 22x22mm coverslips adhered to a slide using gelatin and covered with a 22x50mm coverslip to enable embryos to be rotated and viewed from multiple angles.

2.3.7 Microscopy

Stained embryos were examined using a Zeiss Axiophot II microscope. Images were captured on Kodak Ektachrome Elite 160T film using Axiophot software and digitised using a Nikon Coolscan II slide scanner. All figures were created in Adobe Photoshop 5.0 on Apple Macintosh computers.

2.3.8 Staging of embryos

Embryonic staging is according to Nüsslein-Volhard and Wieschaus (Wieschaus and Nüsslein-Volhard, 1998).

2.4 Results

The expression pattern of the segmentation genes and a number of dorso-ventral polarity genes during sensory nervous system development were examined. Antibodies against most the segmentation genes are readily available but proved more difficult to obtain for the dorso-ventral polarity genes. In those cases where antibodies were unavailable P[GAL4] or P[lacZ] enhancer-trap lines that are expressed in the same pattern as the gene of interest were used to examine the expression pattern of that gene within the sensory nervous system. The use of such lines does carry the caveat however that any expression observed within the sensory nervous system does not necessarily reflect the endogenous expression pattern of that gene and could merely be an artefact of that particular enhancer-trap line. Nevertheless, such lines can prove useful markers of cell fate. Antibodies were also obtained against the protein products of genes known to determine sensory modality. Additionally, the expression pattern of a number of other genes has been examined. These are primarily known cell-surface proteins already implicated in axon guidance or genes already known to be expressed in other sensory structures such as photoreceptors.

2.4.1 Segmentation gene expression in the PNS

2.4.1.1 Overview of segmentation gene expression in the sensory nervous system

The expression pattern of each gene product was examined throughout embryonic development and the appropriate pattern of gap, pair-rule or segment polarity gene expression was observed during early embryogenesis. Figure 2.2 illustrates the typical patterns of segmentation gene activity observed. These expression patterns have been well documented elsewhere and will not be discussed further.

The expression of the segmentation and dorso-ventral gene products in the sensory nervous system is summarised in Table 2.1. Of the 27 genes tested 9 showed patterns of gene activity that potentially included sensory neurons or their precursors and were selected for further study. The remaining 17 were not expressed in the sensory nervous system. These either showed no expression after the initial period of segmentation gene activity or were re-expressed after sensory nervous system development had begun but were not expressed in the sensory nervous system. As an example, the staining achieved with the Even-skipped (Eve) antibody is shown in Figure 2.3. Eve is expressed in a subset of neuroblasts in the ventral nerve cord (Figure 2.3 A, B, arrowhead) but there is no expression in the peripheral nervous

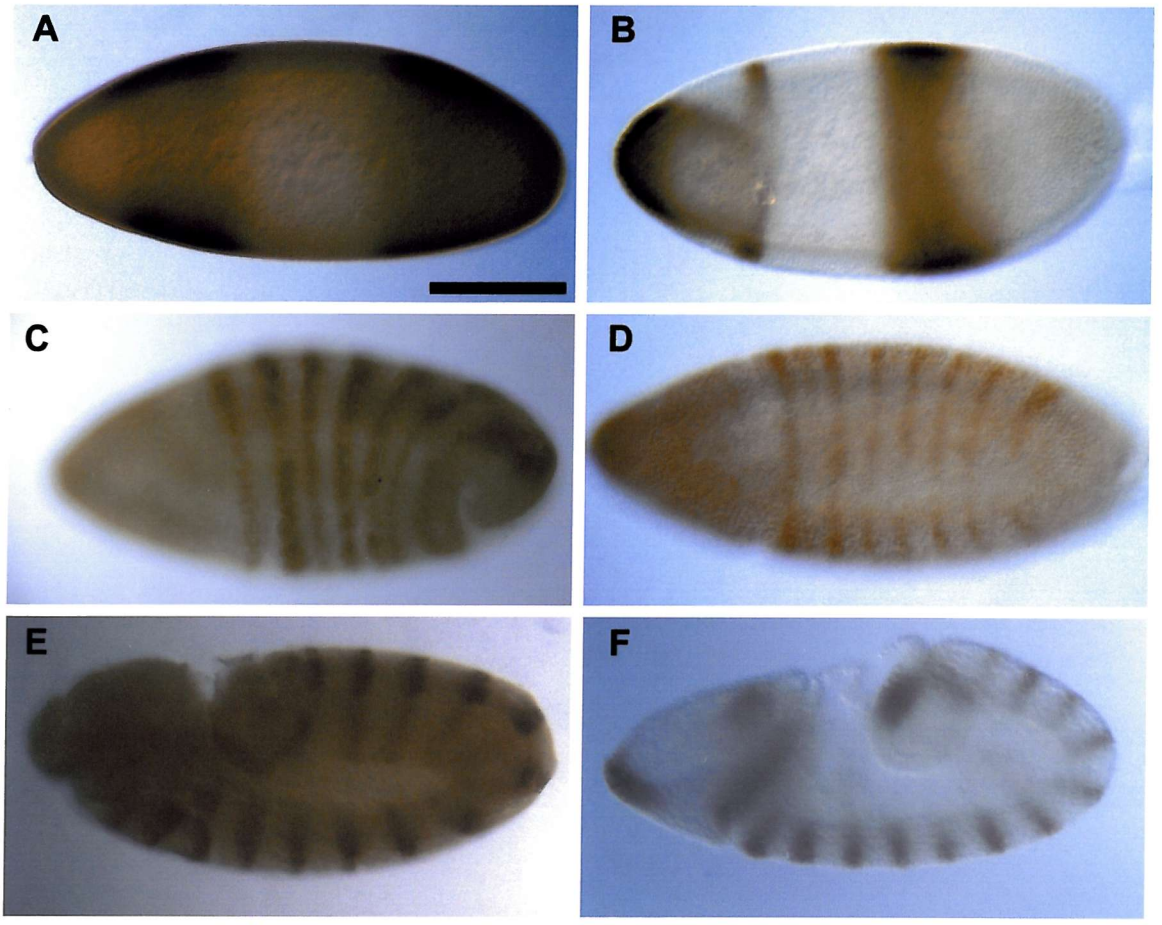


Figure 2.2 Segmentation gene expression. A. Giant Gap gene expression pattern **B.** Knirps Gap gene expression pattern **C.** Fushi-tarazu Pair-rule gene expression pattern. **D.** Runt Pair-rule gene expression pattern. **E.** Engrailed Segment-polarity gene expression pattern **F.** Wingless Segment-polarity gene expression pattern. (A-F) Scale bar 0.1mm.

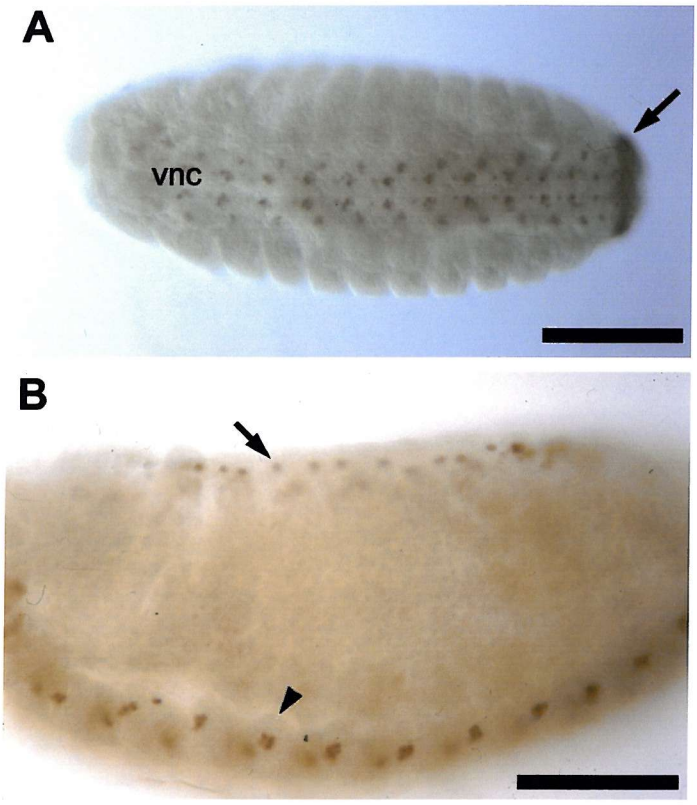


Figure 2.3 Even-skipped expression at stage 16. A. Even-skipped is expressed in a segmentally repeated subset of cells within the ventral nerve cord (vnc) and in the proctoderm (arrow). **B**. Eve is expressed in the paracardial cells (arrow). The arrowhead highlights staining of neuroblasts within the vnc. (A) Scale bar 0.1mm. (B) Scale bar 0.1mm.

system. Eve is also expressed in part of the proctoderm (Figure 2.3 A, arrow) and in a series of cells along the dorsal edge (Figure 2.3 B, arrow). These cells correspond to the paracardial cells, the embryonic heart precursors of *Drosophila.*

The results achieved with the Dishevelled and Hedgehog antibodies produced very weak staining patterns that appeared to indicate no staining of any structures of interest. In these cases, despite experimenting with different antibody dilutions and staining protocols, no significant improvement in the quality of the staining was achieved. The expression of both *dishevelled* and *hedgehog* was therefore also examined using P[GAL4] enhancer-trap lines and while the appropriate pattern of segment-polarity gene expression was observed during early embryogenesis in both cases (data not shown), no expression was observed in the sensory nervous system.

Table 2.1 Segmentation and dorso-ventral gene expression within the PNS

Class of patterning gene	Gene	Potential expression in PNS?
maternal	*bicoid*	No
gap genes	*caudal*	No
	giant	No
	huckebein	Yes
	hunchback	Yes
	knirps	Yes
	kruppel	Yes
	tailless	No
pair-rule genes	*even-skipped*	No
	fushi-tarazu	No
	hairy	No
	odd-skipped	No
	paired	No
	runt	Yes
	sloppy-paired	Yes
segment polarity genes	*armadillo*	Yes
	cubitus-interruptus	No
	dishevelled	No
	engrailed	Yes
	gooseberry	No
	hedgehog	No
	patched	No
	wingless	Yes
dorso-ventral genes	*apterous**	No
	decapentaplegic	No
	*distalless**	No
	twist	No

* determine dorso-ventral axis in imaginal discs

The 9 genes that were potentially expressed in the sensory nervous system included four gap genes (*huckebein*, *hunchback*, *knirps*, and *kruppel*), two pair-rule genes (*runt* and *sloppy-paired*) and two segment polarity genes (*armadillo*, and *wingless*). In order to determine whether the peripheral expression pattern observed for each of these 9 genes did include sensory neurons and/or precursors, double-labelling was performed using an appropriate marker for the sensory nervous system. Mab22c10, which stains the membrane and cellular processes of all sensory neurons (Figure 2.22 A, C) was used to examine the expression of nuclear proteins while Elav, which stains the nuclei of all neuronal cells (Figure 2.22 B, D), was used as a counterstain for non-nuclear proteins. The A37 P[lacZ] enhancer-trap line, which is expressed throughout sensory nervous system development (Ghysen and O'Kane, 1989), serves as a nuclear marker for SOPs and sensory neurons. An antibody against HRP, a membrane-bound marker for the entire nervous system, is available directly conjugated to FITC and was occasionally used for fluorescent detection.

The results are shown in Table 2.2. Of the 9 genes selected for further study, 4 showed expression in differentiated sensory neurons. There were *armadillo*, *engrailed*, *runt*, and the huckebein-lacZ enhancer-trap line. Of the remaining 5, *sloppy-paired* and *wingless* were potentially expressed in peripheral glia and es organ support cells, respectively and 4 were not expressed in the sensory nervous system at any stage during its development.

Table 2.2 Segmentation gene expression in sensory neurons

Gene	Sensory neuron expression
armadillo	Yes
engrailed	Yes
huckebein	Yes
hunchback	No
knirps	No
kruppel	No
runt	Yes
sloppy-paired	No
wingless	No

The results obtained for each of the 9 genes showing potential sensory nervous system expression will now be discussed individually. Expression is described from the period of sensory nervous system formation onwards.

2.4.1.2 Segmentation genes with potential sensory nervous system expression

Armadillo

By stage 15-16 Armadillo expression becomes apparent in a discrete number of cells throughout the periphery (Figure 2.4 A). The pattern of expression revealed by antibody staining indicated that Armadillo expression may be confined to a subset of cells in the peripheral nervous system. Staining was visible within all five lch5 neurons and a number of other peripheral cells (Figure 2.4 B) but too few an amount to include all sensory neurons. Staining also appeared to be localised to the tips of cells (Figure 2.4 B, arrow and arrowheads). This, in conjunction with the punctate nature of the staining pattern, suggested that Armadillo may be specifically expressed in the dendrites of neuronal cells.

In order to clarify the Armadillo expression pattern within the peripheral nervous system, embryos stained for Armadillo were counterstained with antibodies against Elav. Armadillo expression labels all external sensory and chordotonal neurons but no multidendritic neurons. Armadillo expression is also localised to the dendritic regions of external sensory neurons (Figure 2.4 C, arrow) and chordotonal neurons (Figure 2.4 E, F).

Engrailed

Two peripheral cells in each hemisegment continue to express Engrailed throughout embryonic development and, as the epidermal Engrailed stripe fades, the only remaining Engrailed-expressing cells in the periphery are these two cells (Figure 2.5 B, arrow), one of which lies within the Engrailed stripe, and three to four of the lateral chordotonal organ (lch5) neurons (Figure 2.5 C, arrowheads). Expression in the lch5 neurons is transient however and cannot be detected in later embryos (Figure 2.5 C, inset).

To resolve the pattern of expression embryos were double-stained for Engrailed and Mab22c10. The resulting double staining reveals the chordotonal organ clearly emerging from the Engrailed stripe (Figure 2.5 D, E). Engrailed expression can be seen within several of the chordotonal neurons (Figure 2.5 E, white arrow) and the solo lateral Engrailed-positive cells is

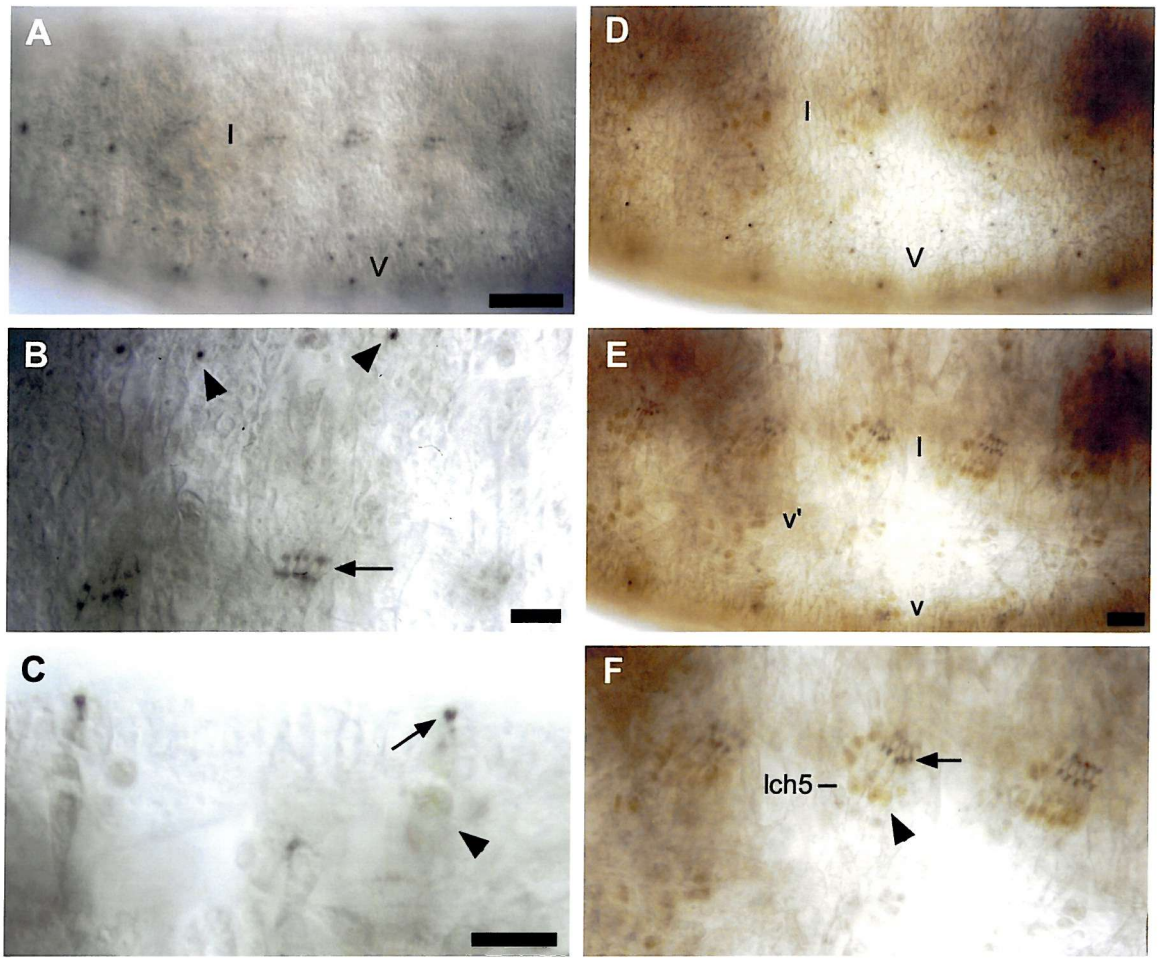


Figure 2.4 Armadillo expression in the PNS. A-B, Armadillo expression. C-F, Armadillo/Elav expression. Armadillo staining is black. Elav staining is brown. **A.** Armadillo expression in the periphery. **B.** Armadillo is expressed in the lateral chordotonal organ (arrow), and in a punctate pattern in a number of other peripheral cells (arrowheads). **C.** The dendrite of an es neuron stained with armadillo can be seen projecting into the epidermis (arrow). The arrowhead indicates Elav staining in the nucleus of the es neuron. **D.** Armadillo/Elav expression in the periphery. **E.** Armadillo/Elav expression in the periphery, showing expression in lch5. **F.** Shows the armadillo staining within lch5 in greater detail. The arrow highlights the armadillo staining in the scolopidial cells of the chordotonal organ, into which the dendrites of the chordotonal neurons project, and the arrowhead highlights the Elav staining of the nuclei of the lch5 neurons. v - ventral, v' - ventro-lateral, l - lateral, d - dorsal. (A-B) Scale bar 0.03mm. (C) Scale bar 0.01mm. (D) Scale bar 0.01mm. (E-F) Scale bar 0.01mm.

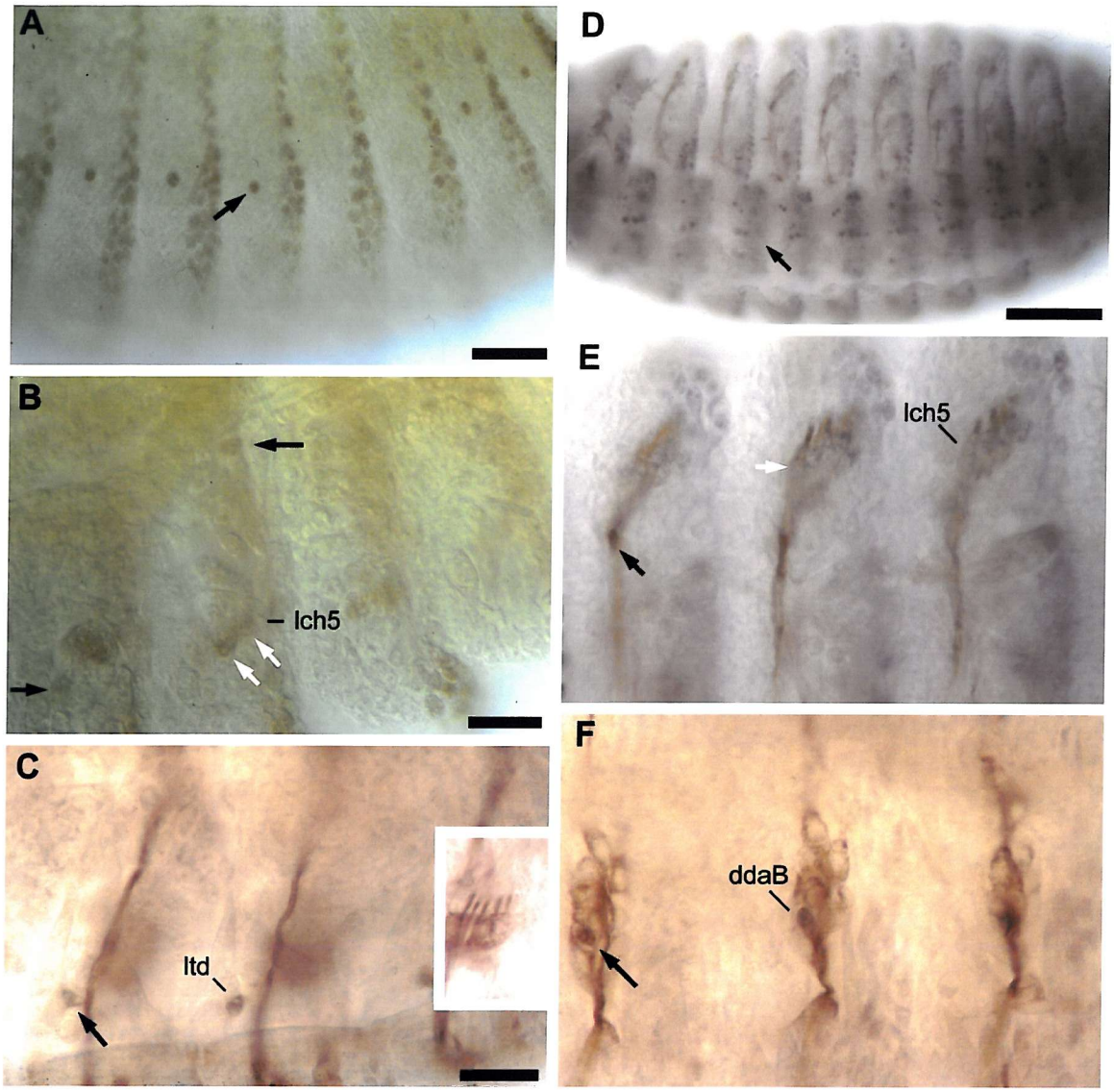


Figure 2.5 Engrailed expression in the PNS. A-B, Engrailed expression. C-F, Engrailed/Mab22c10 expression. Engrailed staining is black. Mab22c10 staining in brown. **A.** An epidermal stripe of engrailed expression is visible in each segment. A single cell that lies clear of the engrailed stripe is also stained (arrow). **B.** Transient expression is detected in three to four lch5 neurons (white arrows) as the stripe of engrailed expression fades. Staining is also visible in a lateral cell, slightly ventral and anterior to lch5 (black arrow), and a dorsal cell (black arrow). **C.** Engrailed is expressed in ltd. Inset shows that no engrailed expression is detected in the lch5 neurons once the sensory nervous system is fully formed. **D.** Lateral view showing Engrailed/22c10 peripheral expression. Engrailed is also expressed in a segmentally repeated array of neuroblasts within the ventral nerve cord (arrow). **E.** Shows the lateral chordotonal neurons emerging from the stripe of engrailed expression. The white arrow highlights the nuclear engrailed staining within the chordotonal neurons. The black arrow highlights expression in ltd. **F.** Engrailed is expressed in the ddaB dorsal cluster neuron (arrow). (A) Scale bar 0.03m. (B) Scale bar 0.01mm. (C, E-F) Scale bar 0.015mm. (D) Scale bar 0.06mm.

visible at the base of the lch5 (Figure 2.5 E, black arrow). The two cells that continue to express Engrailed throughout embryogenesis can be identified as one of the dorsal cluster multidendritic neurons, ddaB (Figure 2.5 F, arrow) and the lateral tracheal dendritic neuron, ltd (Figure 2.5 C, arrow).

Huckebein

Huckebein (Hkb) expression was examined using two different P[lacZ] enhancer-trap lines, hkb-lacZ and hkb-lacZ A17, which both produce similar staining patterns. At stage 15 expression is observed in a discrete number of peripheral cells immediately adjacent to the ventral nerve cord (Figure 2.6 B, arrows and arrowheads) and a group of lateral cells (Figure 2.6 C, arrow). All the cells labelled in the periphery are weakly labelled.

Embryos were double-stained with Mab22c10 and anti-β-galactosidase to determine the identity of the lacZ-expressing cells. Huckebein-lacZ expression is apparent in the neurons of the lateral chordotonal organ lch5 (Figure 2.6 D, black arrows), the ventral chordotonal neurons vchA and vchB (Figure 2.6 E, arrowheads), and within some, possibly all, neurons of the ventral and ventro-lateral clusters of sensory neurons (Figure 2.6 E, F). As this expression pattern includes more lacZ-positive cells than originally observed in embryos stained for hkb-lacZ alone, some cross reaction between the Mab22c10 staining and the anti-β-galactosidase staining may be occurring and the increased number of neurons that express hkb-lacZ could be an artefact.

The co-localisation was therefore repeated using fluorescently labelled secondary antibodies. Embryos were double-stained for β-galactosidase and HRP. Hkb-lacZ expression was still visible in the ventral nerve cord, where it labels a segmentally repeated subset of neuroblasts, but not in the periphery (data not shown). It would appear that hkb-lacZ is not expressed at a high enough level in the sensory nervous system to be detected using fluorescent secondary antibodies which are not as sensitive as detection using biotinylated antibodies. As expression in a lateral group of neurons in a position consistent with being lch5 neurons was observed when embryos were stained for hkb-lacZ alone, it seems likely that the staining of the lch5 neurons in embryos double-stained for hkb-lacZ and Mab22c10 is genuine. As the expression of huckebein-lacZ in the ventral and ventro-lateral groups of sensory neurons has not been reliably determined, hkb-lacZ cannot therefore be considered to label these sensory neurons.

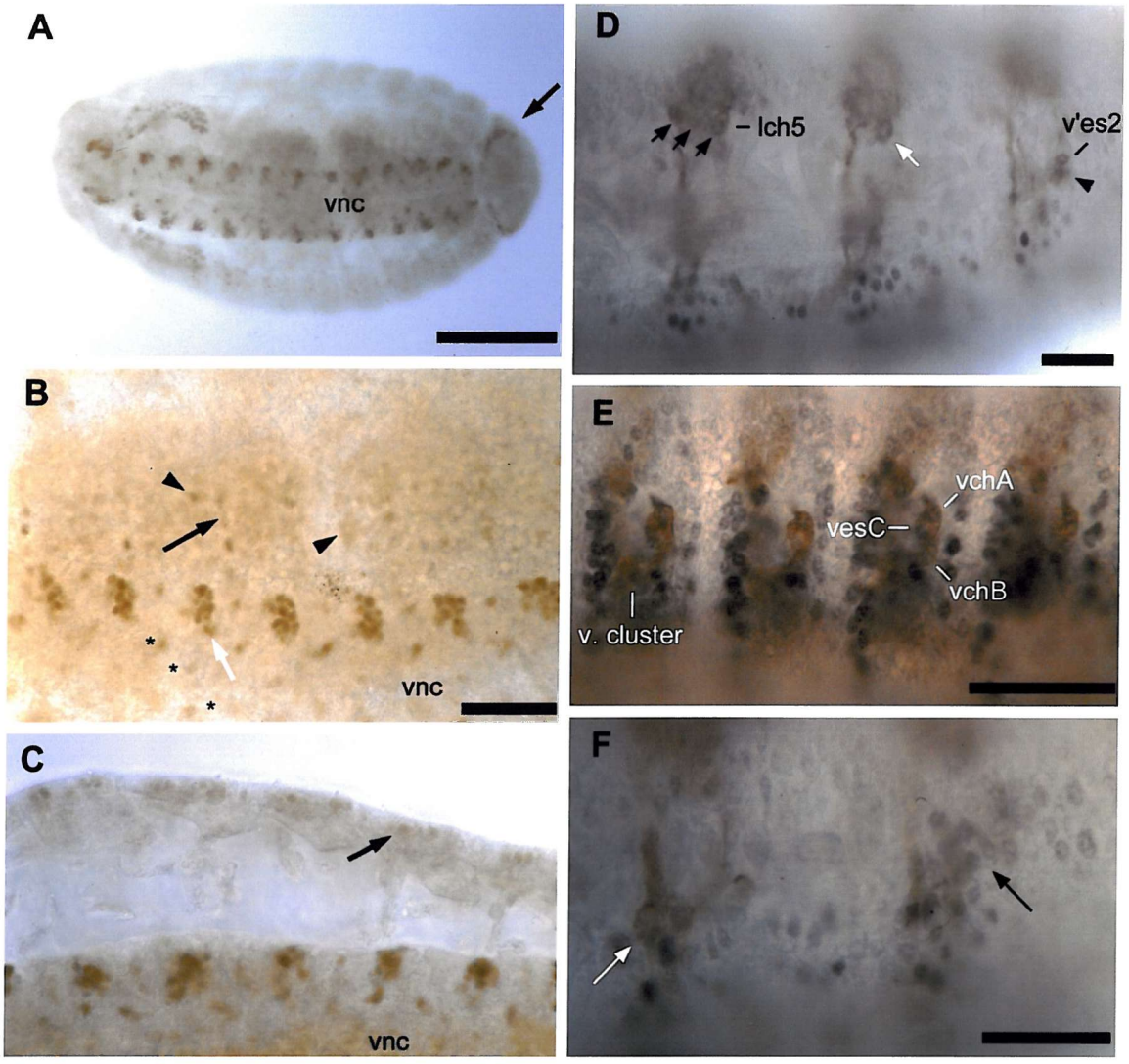


Figure 2.6. Huckebein expression in the PNS. A-C, Huckebein-lacZ expression. D-F, Huckebein-lacZ/Mab22c10 expression. Staining for β-galactosidase is black. Mab22c10 staining is brown. **A.** Ventral view showing huckebein-lacZ expression in the ventral nerve cord and in a posterior band of cells (arrow). **B.** Huckebein is expressed in a subset of CNS neurons and neuroblasts (white arrow and asterisks). There is also weak expression in the ventral region of each segment, where huckebein-lacZ labels a line of cells and a number of discrete cells in each hemisegment. **C.** Huckebein-lacZ expression is also detected in a group of lateral cells (arrow). **D.** Huckebein-lacZ staining is observed in the lch5 neurons (black arrows), in a neuron underlying the lch5 organ (white arrow) and in v'es2 and v'pda (arrowhead). **E.** Huckebein-lacZ expression is detected in the ventral cluster md neurons, vesC, vchA, and vchB. **F.** Huckebein-lacZ staining is also detected in two further groups of ventro-lateral neurons, which correspond to the v'ada/v'esB (white arrow) and the v'td2/ v'esA (black arrow) neurons. (A) Scale bar 0.1mm. (B-C) Scale bar 0.03mm. (D) Scale bar 0.01mm. (E) Scale bar 0.01mm. (F) Scale bar 15μm.

Hunchback

At stage 13, after germ band shortening, Hunchback expression begins to be detected in a discrete number of cells throughout the ventro-lateral region of each abdominal segment (Figure 2.7 A, B, arrows) and in a cluster of approximately eight to ten cells in the dorso-lateral region of each segment (Figure 2.7 A, arrowhead and Figure 2.7 C). These cells are located in a similar position to the cells of the sensory nervous system. Expression in these cells is transient however and by stage 15 Hunchback expression is no longer detectable in the periphery (Figure 2.7 D).

Double-staining embryos for both Hunchback and HRP showed that Hunchback expression in peripheral cells precedes HRP expression in sensory neurons (Figure 2.8 A, C). Hunchback is therefore not expressed in differentiated sensory neurons but could label sense organ precursor cells. Hunchback expression was examined in the A37 P[lacZ] enhancer-trap line to determine whether Hunchback labels sense organ precursors. No co-localisation was detected (Figure 2.8 A, C), indicating that Hunchback is not expressed in the sensory nervous system at any stage.

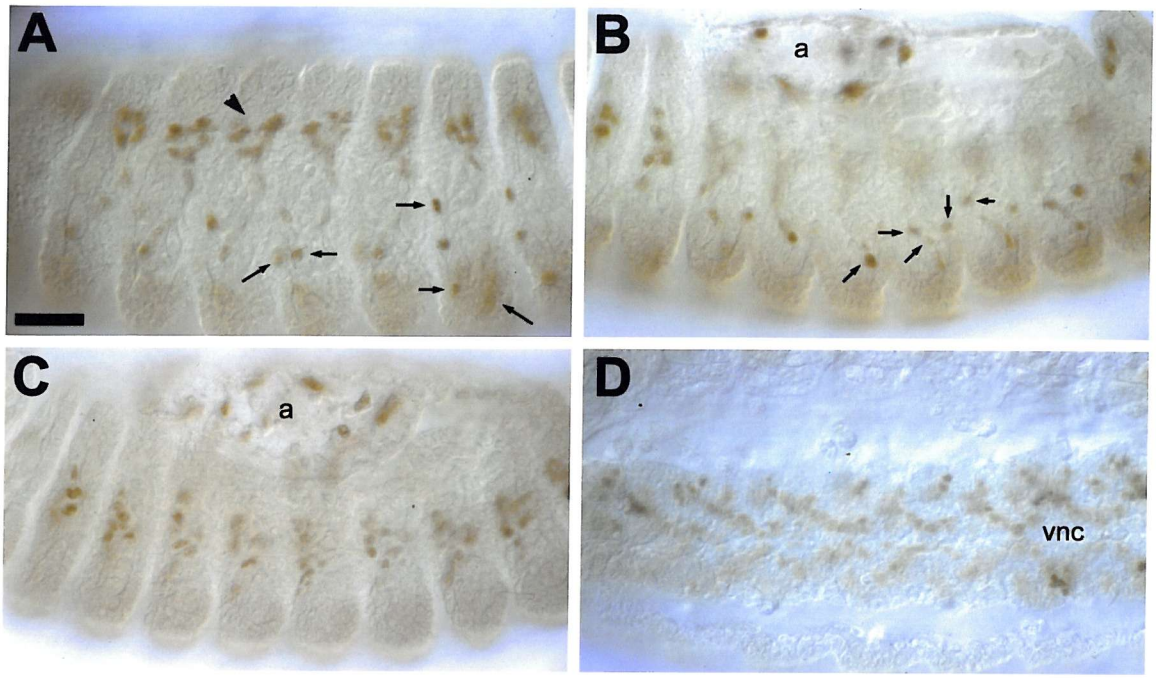
Knirps

In the dorsal region of each segment there is a thin strip of Knirps expressing cells (Figure 2.9 B, arrow). In the lateral region of each segment a cluster of cells is stained (Figure 2.9 B, arrow), and in the ventral region a number of discrete cells are labelled (Figure 2.9 C, arrows). These groups of cells occupy positions that potentially overlap with the sensory nervous system.

To test this possibility, embryos were double-stained for Knirps and Mab22c10. This showed that Knirps is not expressed in any dorsal sensory neurons but did not completely resolve Knirps expression in the rest of the sensory nervous system. The dorsal cluster of sensory neurons occupies a position above the lateral cluster of Knirps-positive cells but below the dorsal stripe of Knirps expression (Figure 2.9 E, arrow) but the lateral and ventral clusters of Knirps-expressing cells overlie much of the remainder of the sensory nervous system (Figure 2.9 F, G). While Knirps staining overlaps lch5, v'ch1, vchA and B, all the chordotonal neurons are internal to the Knirps-positive cells and do not express Knirps (Figure 2.9 F). The superficial location of the cells that express Knirps suggests that Knirps may only label epidermal cells but the extent of the Knirps expression within the epidermis precludes the

Figure 2.7 Hunchback Expression. A-C, Hunchback expression in a stage 13 embryo. D, Hunchback expression in a stage 16 embryo. **A.** Hunchback is expressed in a cluster of peripheral cells in the dorso-lateral region of each hemisegment (arrowhead) and a number of single cells throughout the ventro-lateral region (arrows). **B.** A slightly different plane of focus reveals staining of several additional cells in the ventral region (arrows). Hunchback staining is also evident within the amnioserosa (a). **C.** A more dorsal view of the staining in the dorsolateral cluster and the amnioserosa. **D.** Ventral view of a stage 16 embryo showing Hunchback expression within the ventral nerve cord (vnc). By this stage expression is no longer visible in the periphery. (A-D) Scale bar 0.03mm.

Figure 2.8 Hunchback expression in the PNS. A, C, Hunchback/HRP double labelling. Hunchback staining is red. HRP staining is green. B, D. Hunchback/A37 P[lacZ] double labelling. Hunchback staining is red. β-galactosidase staining is green. **A.** Hunchback and HRP expression do not co-localise in the periphery. **B.** Hunchback and A37 P[lacZ] do not co-localise in the periphery. A band of β-galactosidase expression in visible in cephalic segments (arrow) **C.** Hunchback labels neuroblasts in the vnc (arrows). **D.** Hunchback expression in the amnioserosa. (A-D) Scale bar 0.1mm.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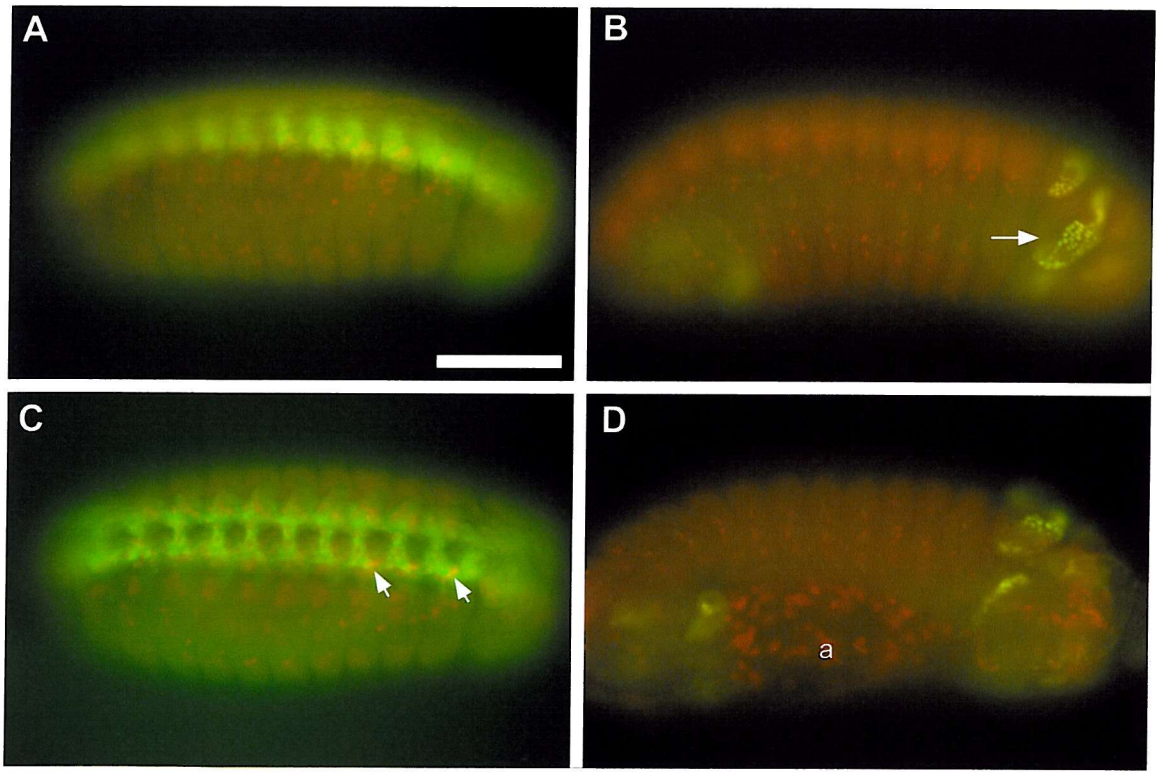
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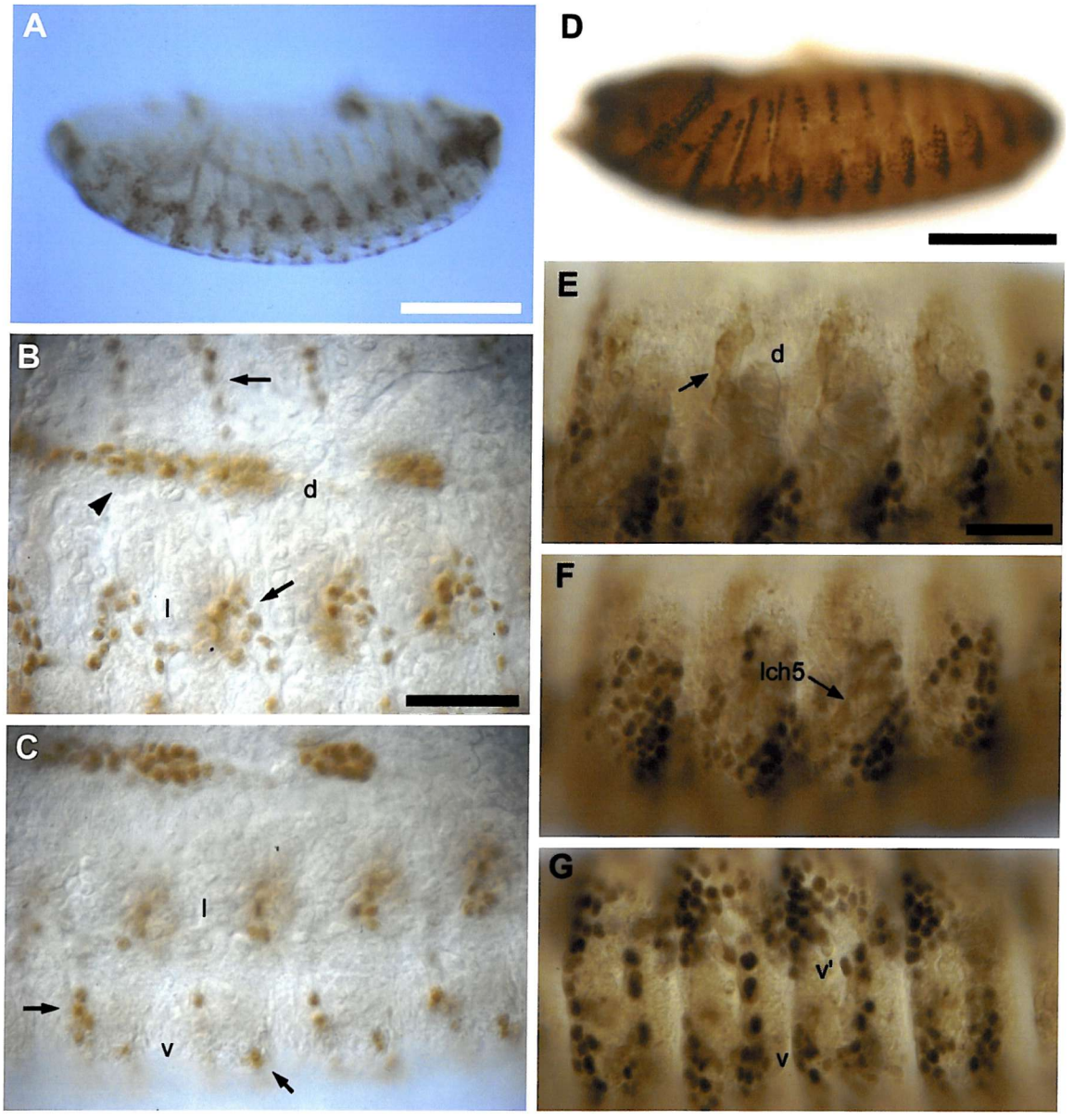


Figure 2.9 Knirps Expression in the PNS. A-C, Knirps expression. D-G, Knirps/Mab22c10 expression. Knirps staining is black. Mab22c10 staining in brown. **A.** Lateral view showing Knirps expression in the periphery. **B.** Knirps is expressed in a dorsal stripe of cells (arrow) and a lateral cluster of cells (arrow). A longitudinal stripe of cells, located more internally, is also stained (arrowhead). **C.** Knirps also stains a number of discrete cells in the ventral region (arrows). **D.** Knirps/Mab22c10 staining in the periphery. **E.** Sensory neurons in the dorsal region lie clear of the Knirps-expressing cells. Arrow indicates the dorsal cluster of sensory neurons. **F.** Knirps is not expressed in the lch5 neurons (arrow). **G.** Knirps expression obscures the sensory neurons in the ventro-lateral and ventral regions of the embryo. d - dorsal, l -lateral, v - ventral. (A) Scale bar 0.1mm. (B-C) Scale bar 0.03mm. (D) Scale bar 0.1mm. (E-G) Scale bar 0.03mm.

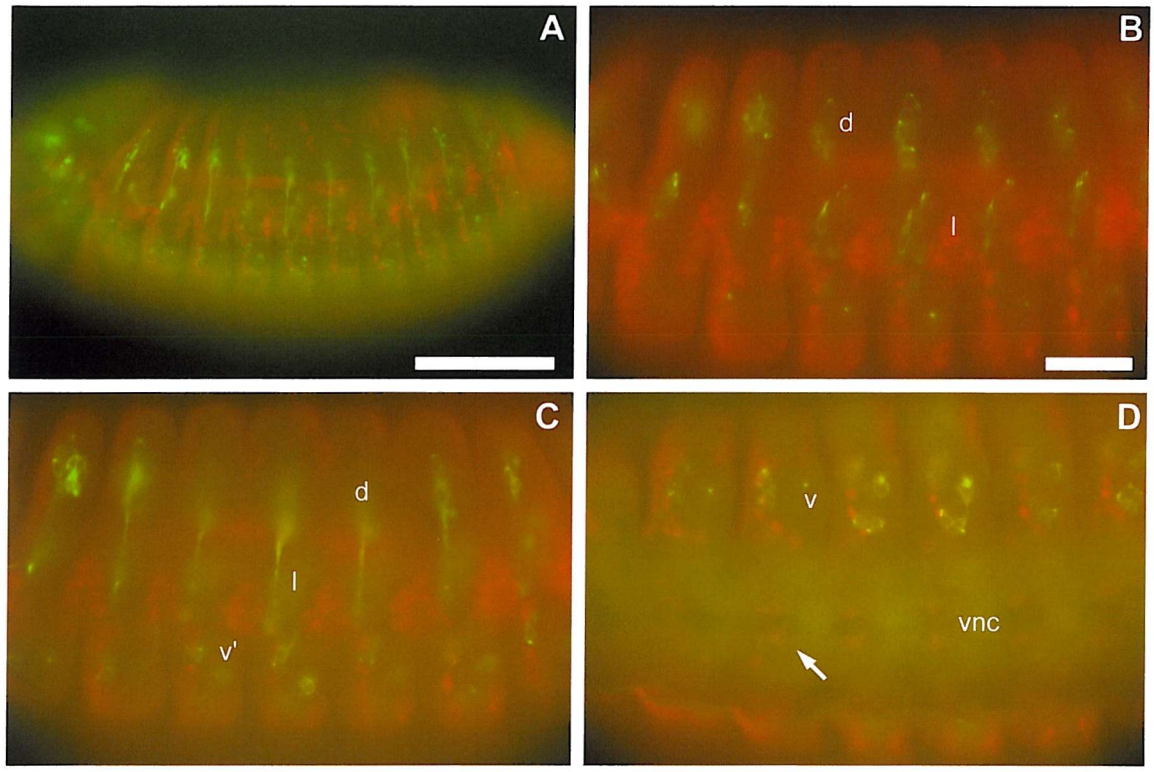


Figure 2.10 Fluorescent Detection of Knirps/mab22c10 Expression in the PNS. Knirps staining is red. Mab22c10 staining is green. **A.** Lateral view showing Knirps/Mab22c10 staining in the periphery. **B.** Knirps staining does not co-localise with Mab22c10 in the dorsal and lateral region. **C.** Knirps staining does not co-localise with Mab22c10 in the ventro-lateral region. **D.** Knirps staining does not co-localise with Mab22c10 in the ventral region. Arrow indicates Knirps expression in a subset of CNS cells. (A) Scale bar 0.1mm. (B-D) Scale bar 0.03mm.

visualisation of all neurons. The possibility that a number of external sensory neurons are labelled cannot be excluded.

The double-staining of embryos for Knirps and Mab22c10 was therefore repeated using fluorescent secondary antibodies. The fluorescent visualisation of the sensory nervous system revealed that all sensory neurons are produced from regions of the periphery that are closely juxtaposed to areas of Knirps protein expression, but also showed that Knirps does not co-localise with Mab22c10 within the sensory nervous system (Figure 2.10).

Kruppel

Kruppel is re-expressed during germ band extension (stage 11) in a distinct subset of peripheral cells in each segment (Figure 2.11 A). At this point the SOPs are being singled out from the proneural clusters and the cells that express Kruppel could correspond to the peripheral sense organ precursors. At stage 13, after the completion of germ band shortening, Kruppel expression persists in a subset of peripheral cells (Figure 2.11 B). Clusters of Kruppel-expressing cells are observed in positions that closely mirror the locations of the ventral, ventro-lateral, lateral and dorsal clusters of sensory neurons in the sensory nervous system (Figure 2.11 D, E). The linear arrangement of the dorsal group of Kruppel-positive cells in particular reflects the organisation of the dorsal cluster of sensory neurons. By stage 15, when the sensory nervous system is fully formed, Kruppel is no longer detectable in the periphery in any cells except tracheae (Figure 2.11 C, arrows).

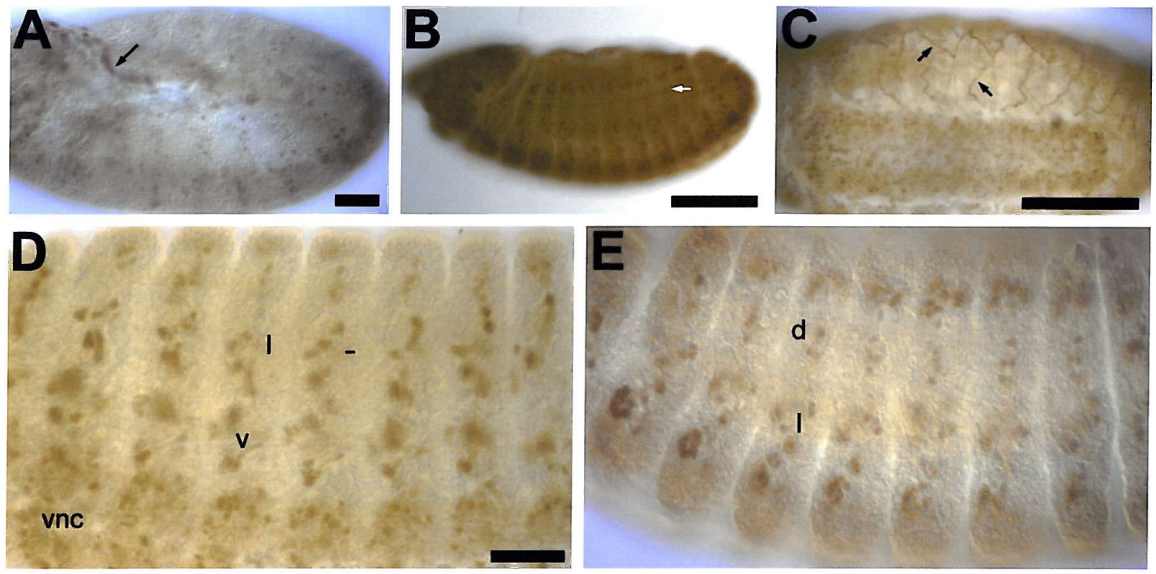
Kruppel expression was examined in the A37 P[lacZ] enhancer-trap line to determine whether the cells detected with the Kruppel antibody are sense organ precursors. Double-labelling revealed that, while the Kruppel expressing cells often occur in close association with the sensory nervous system, Kruppel does not appear to be expressed within the developing sensory nervous system (Figure 2.12). While there was no observed overlap between Kruppel and A37 P[lacZ] expression in any thoracic or abdominal segments, Kruppel and A37 P[lacZ] do co-localise within one of the cephalic sense organs (Figure 2.12 inset), which forms part of the antennomaxilliary complex.

Runt

At stage 13, after germ band shortening, Runt staining is evident in a restricted pattern of peripheral cells in the dorsal and ventral region of each hemisegment. No expression is visible

Figure 2.11 Kruppel Expression. A. Kruppel is re-expressed during germ band extension in a segmentally repeated array of peripheral cells. Expression is also visible in the amnioserosa (arrow). **B.** Lateral view showing Kruppel expression in the periphery in a stage 13 embryo. Arrow indicates tracheal expression. **C.** Ventral view of a stage 15 embryo. Kruppel expression labels trachea in the periphery and a large number of neuroblasts in the vnc. **D.** At stage 13, Kruppel labels a stereotyped pattern of peripheral cells in each segment. **E.** A more dorsal view, showing the Kruppel-expressing cells in the dorsal region of each segment. (A) Scale bar 0.03mm. (B) Scale bar 0.1mm. (C) Scale bar 0.1mm. (D-E) Scale bar 0.3mm.

Figure 2.12 Fluorescent Detection of Kruppel/A37 P[lacZ] Expression in the PNS. Kruppel staining is green. β-galactosidase staining is red. **A** and **C** show the peripheral expression of Kruppel and β-galactosidase at slightly different angles. Inset shows that Kruppel and A37 P[lacZ] expression co-localises within an anterior sense organ (arrow). **B.** Shows the dorso-lateral region of the embryo in greater detail. Kruppel and A37 P[lacZ] expression does not co-localise. Kruppel staining is visible in the amnioserosa (a). **D.** Shows the ventro-lateral region of the embryo in greater detail. Kruppel and A37 P[lacZ] expression does not co-localise. (A,C) Scale bar 0.1mm. (B, D) Scale bar 0.3mm.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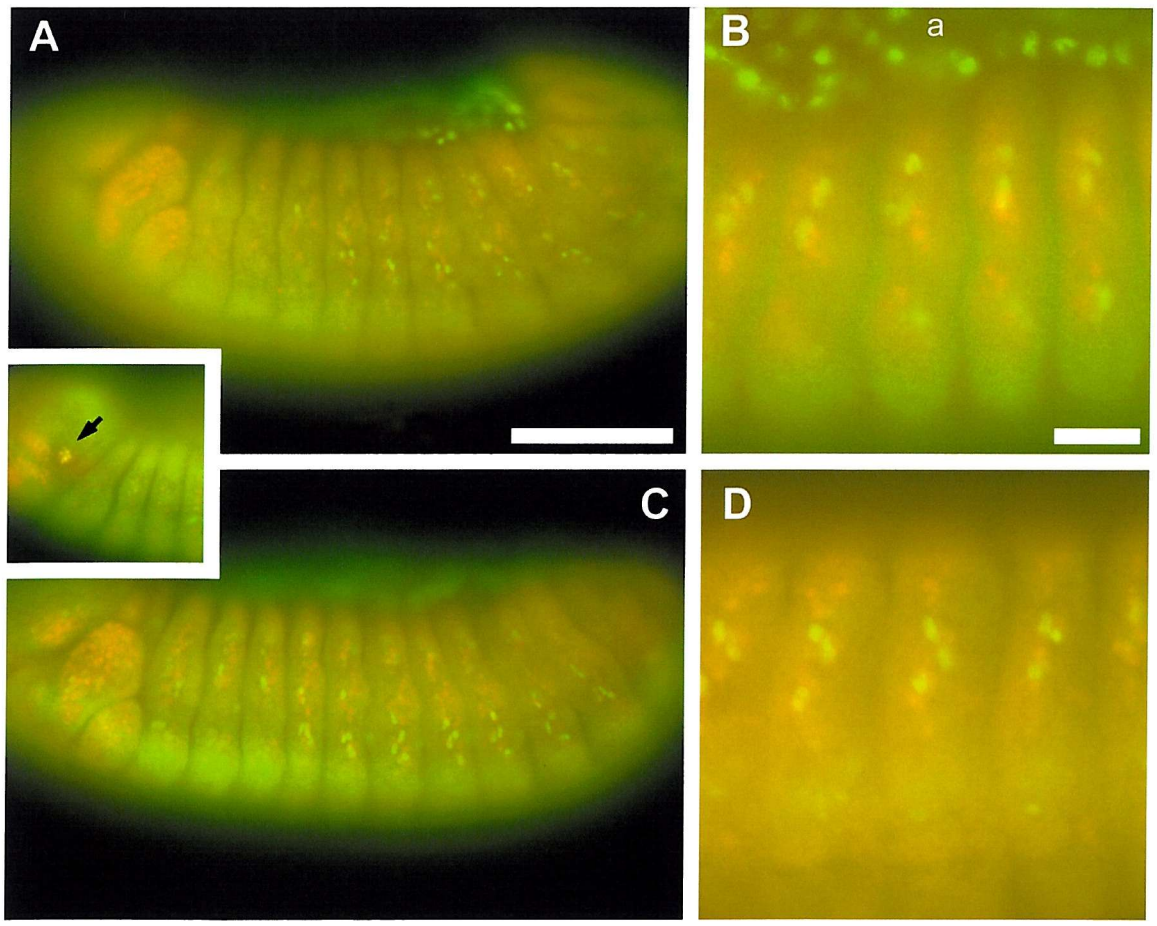
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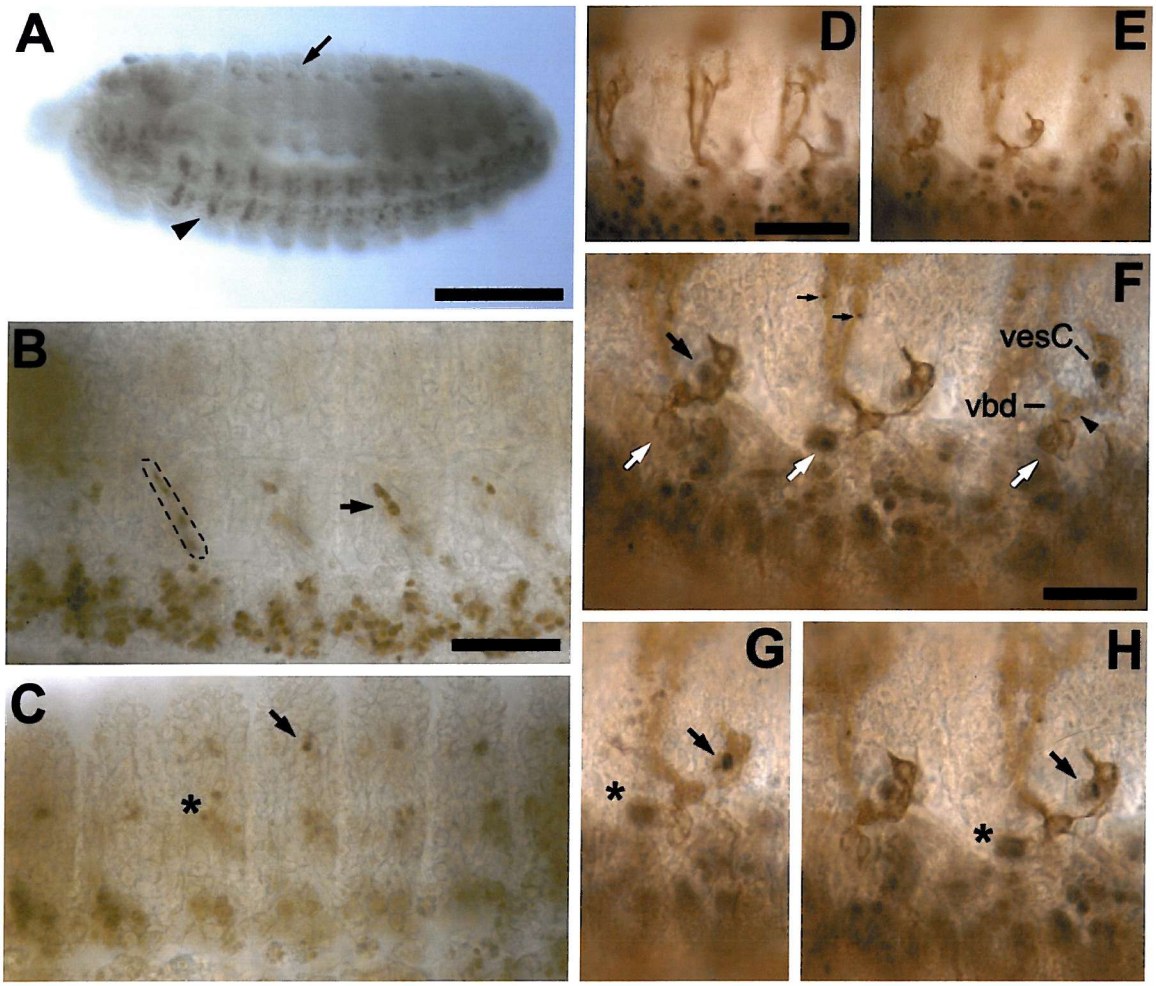


Figure 2.13 Runt Expression in the PNS. A-C, Runt expression. D-H, Runt/Mab22c10 expression. Runt staining is black. Mab22c10 staining is brown. **A.** Lateral view showing runt peripheral expression. The arrow highlights runt expression within the nuclei of one of the dorsal muscles. Runt expression is also visible within a segmentally repeated array of neuroblasts and neurons in the CNS (arrowhead). **B.** Runt expression is detected within the nuclei of a ventral muscle (arrow). A broken line indicates the outline of the runt-expressing muscle in one segment. **C.** Runt is expressed in a cluster of cells in the ventral region (asterisk) and a slightly more lateral cell in a very superficial location (arrow). **D** and **E** show Runt and Mab22c10 expression in the ventral region in two different planes of focus. **F.** Runt is expressed in lesC (black arrow), a number of ventral cluster md-da neurons (white arrows) and vbd (arrowhead). The two spots of Runt staining that are detected on the peripheral nerves (small arrows) are an artefact as Runt is confined to the nuclei of expressing cells. **G** and **H** show the ventral region in a more superficial plane of focus. The Runt-expressing cells are denoted as in **C**, for comparison. (A) Scale bar 0.1mm. (B-C) Scale bar 0.3mm. (D-E) Scale bar 0.3mm. (F-H) Scale bar 15μm

in the intervening lateral region. In the dorsal region a diagonal stripe of Runt staining is visible in each hemisegment (Figure 2.13 A, arrow) within one of the dorsal muscles. One of the ventral muscles also expresses runt (Figure 2.13 B, arrow). In the ventral region Runt also labels a more superficial patch of cells that overlie the runt-expressing muscle (Figure 2.13 C, asterisk). A second cluster of cells is faintly stained in a slightly more ventro-lateral position, including a single strongly stained but very superficial Runt-expressing cell (Figure 2.13 C, arrow).

To determine the identity of the Runt-expressing cells embryos were double-stained for both Runt and Mab22c10. The single slightly ventro-lateral Runt-expressing cell can be identified as the external sensory neuron vesC (Figure 2.13 F, G, H, black arrow), consistent with its superficial location. The more ventral Runt expression labels two ventral cluster md-da neurons (Figure 2.13 F, white arrows; Figure 2.13 G, H, asterisk), which appear to be vdaB and vdaC, and vbd (Figure 2.13 F, arrowhead).

Sloppy-paired

At stage 15 Sloppy-paired (Slp) is expressed in a very discrete pattern of cells in the periphery (Figure 2.14 A). A small number of cells are faintly stained in stereotyped locations in each hemisegment, raising the possibility that Slp could be expressed in only a subset of sensory neurons (Figure 2.14 A, arrows).

Double-staining embryos with Mab22c10 and Slp showed that, while Slp-positive cells are in close association with the sensory nervous system and with the peripheral nerves in particular, Slp is not actually expressed within the sensory neurons themselves (Figure 2.14). Slp-expressing cells occur at branch points in the peripheral nerves and these positions are strongly reminiscent of the locations of peripheral and exit glia. Slp-positive cells are located at the positions where the segmental nerve (SN) splits, with one branch continuing dorsally to serve the majority of the v' region neurons and one branch travelling posteriorly to serve the ventral region neurons (Figure 2.14 B, arrows). Again, where this dorsal branch turns and bends away from the intersegmental nerve (ISN) in a more posterior direction Slp-expressing cells are found (Figure 2.14 B, arrow). A further Slp-positive cell is also visible on the ISN slightly ventral to lch5, mirroring the location of the peripheral glia (Figure 2.14 D, arrow). Slp is also expressed in a cell closely associated with dbd, again a known peripheral glial cell location (Figure 2.14 C, arrow). Two Slp-positive cells also occur at the point where the two main peripheral nerves, the ISN and SN, leave the central nervous system, the location where

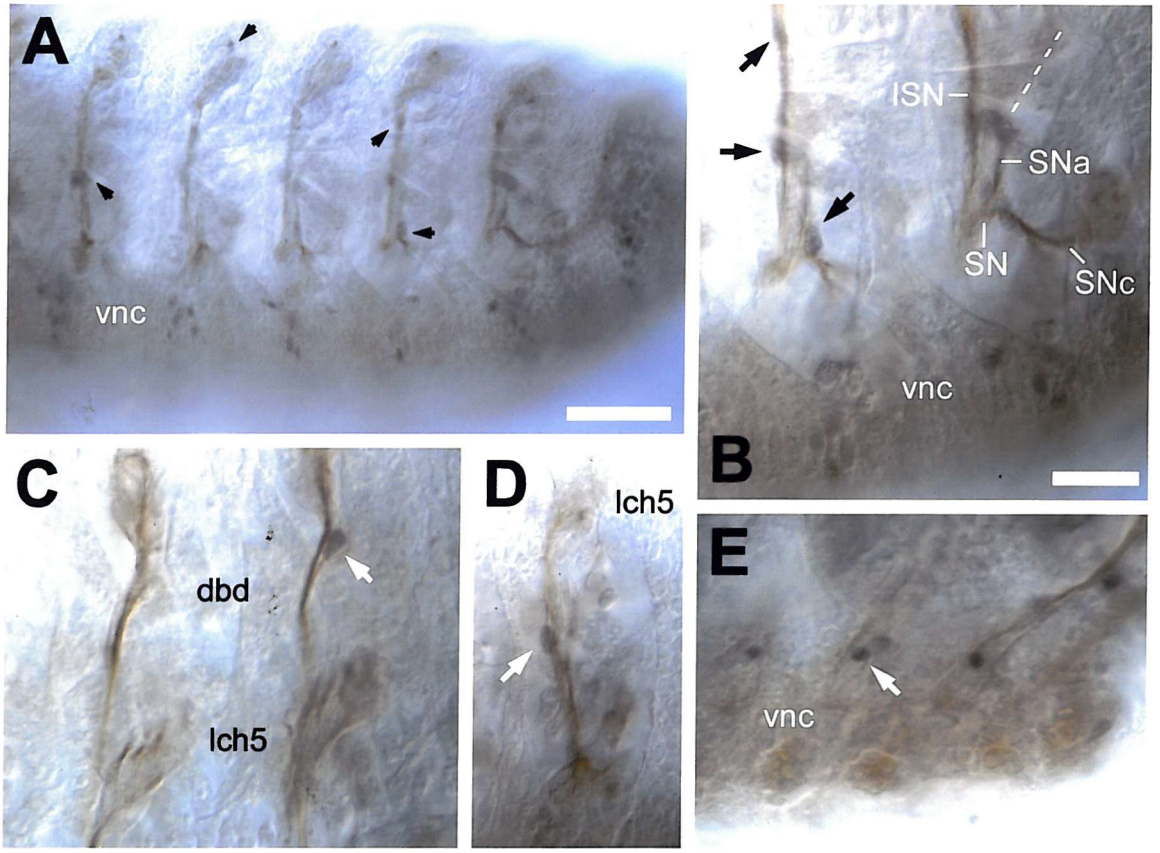


Figure 2.14 Sloppy-paired Expression in the PNS. Sloppy-paired (Slp) staining is black. Mab22c10 staining is brown. **A.** Lateral view showing Slp expression in the periphery. Arrows highlight the locations of the Slp-expressing cells. **B.** Slp is expressed in a number of cells in close association with the peripheral nerves (arrows). Broken line indicates the path of SNa, which continues dorsally. **C.** Slp labels a peripheral cell in close proximity to dbd (arrow). **D.** Shows Slp expression in a lateral cell associated with the ISN (arrow). **E.** Slp labels cells in close proximity to the peripheral nerves as they exit the CNS (arrow). (A) Scale bar 0.03mm. (B-E) Scale bar 15μm.

the cell bodies of the exit glia that ensheath the peripheral nerves are also found (Figure 2.14 E, arrow).

Wingless

As the epidermal stripes of Wingless expression starts to fade, two dorsally located cells that are much more darkly stained than their neighbours are visible within each stripe (Figure 2.15 B). After the initial Wingless pattern of epidermal expression is no longer detectable these two dorsal cells continue to express Wingless.

To identify these cells, embryos were double-stained for Wingless and Mab22c10. Wingless staining is visible in two cells in the dorsalmost region of each segment that appear to match the two previously identified Wingless-expressing cells (Figure 2.15 C white arrows). As these two cells are dorsal to the dorsal cluster, it is possible that they correspond to desC and desD which are the only two neurons that are dorsal to the dorsal cluster. The 22c10 staining in this area was weak and difficult to interpret so it cannot be confirmed that these cells are neuronal. Additional Wingless staining was observed within the dorsal cluster itself (Figure 2.15 C, black arrow) and dbd (Figure 2.15 D). From the staining pattern observed with Wingless alone it is likely that the two wingless-positive cells situated dorsal to the dorsal cluster correspond to the Wingless expressing cells and that the dorsal cluster and dbd expression is simply an artefact.

To resolve the identity of the Wingless-expressing cells the co-localisation was repeated using fluorescently labelled secondary antibodies and embryos were double-stained for Wingless and Elav. The Wingless-expressing cells are indeed dorsal to the dorsal cluster (Figure 2.16 A, C arrows) and the ventralmost of these cells lies directly between desC and desD and borders both cells while the dorsalmost cell is considerably dorsal to desD (Figure 2.16 C). This clearly showed that, while at least one of the Wingless-expressing cells is closely juxtaposed with the dorsalmost external sensory neurons, Wingless is not expressed in sensory neurons. Wingless may instead label external sensory organ support cells and, from the similarity of the location of the wingless-positive cells to the location of the es organ support cells labelled by Neurotactin (Figure 2.25), it seems likely that wingless might be expressed in either the tricogen or the tormogen cells of the es organ.

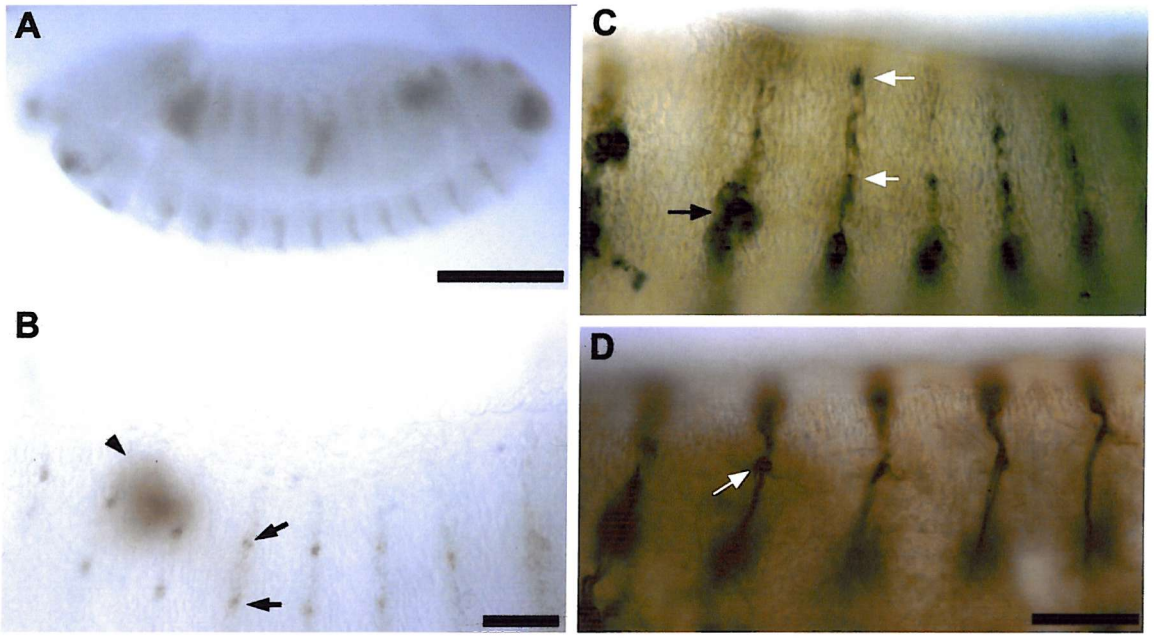


Figure 2.15 Wingless expression in the periphery. A-B, Wingless expression. C-D, Wingless/Mab22c10 expression. Wingless staining is black and Mab22c10 staining is brown **A.** Each stripe of wingless expression splits into a distinct ventral and dorsal region. **B.** As the stripe of wingless expression fades, two cells per segment are left expressing wingless in the dorsal region (arrows). Arrowhead indicates internal wingless expression. **C.** There are two wingless expressing cells in the dorsal-most region of the segment (white arrows). These could correspond to desC and desD, but the 22c10 neuronal staining in this region is ambiguous. There also appears to be wingless staining (black arrow) in the dorsal cluster. **D.** Possible wingless staining is also apparent in the dorsal neuron dbd. (A) Scale bar 0.1mm. (B-C) Scale bar 0.03mm/ (D) Scale bar 0.3mm.

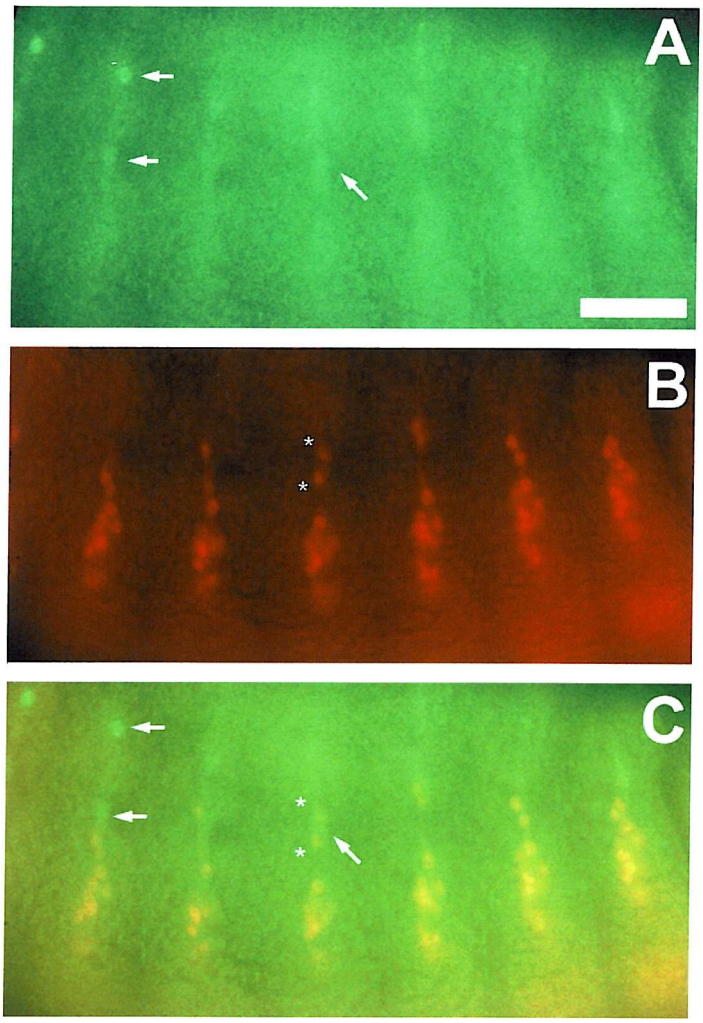


Figure 2.16 Fluorescent detection of Wingless /Elav expression in the PNS. Wingless staining is green. Elav staining is red. **A.** Shows Wingless expression in two cells in the dorsal region (arrows). **B.** Shows Elav expression in the PNS. Asterisks indicate the position of the dorsal external sensory neurons desC and desD . **C.** Wingless does not co-localise with Elav in sensory neurons. Asterisks indicate desC and desD and arrows highlight Wingless expression. (A-C) Scale bar 0.03mm.

2.4.2 Expression patterns of genes that confer sensory modality

The expression patterns of a number of genes known to confer aspects of sensory neuron identity were also examined. Such genes provide potentially useful markers of cell fate and their expression pattern within the sensory nervous system is summarised in Table 2.3, below.

Table 2.3 Genes that determine sensory modality

Class of gene	Gene	Expression pattern in PNS
proneural	*atonal*	ch precursors
identity selector	*BarH1*	es organs (neuron and thecogen)
	BarH2	es organs (neuron and thecogen)
	cut	es organs (all cells) and precursors
	pox-neuro	es neurons

The expression pattern obtained with each antibody will be reviewed in the following section.

Atonal

Atonal is a proneural gene and is required for the formation of a subset of sense organ precursors, the chordotonal organ precursors. Atonal is expressed specifically in neuronal precursors during germ band extension and is not expressed at any other point during embryonic development.

Initially, Atonal staining is visible in patches of epidermal cells surrounding the regions where the chordotonal organ precursors will delaminate from the epithelium during early germ band extension (Figure 2.17 A, arrow). This expression becomes restricted to single cells and five chordotonal precursor cells per abdominal segment retain Atonal expression and become neural precursors (Figure 2.17 B, arrowheads). In later germ band extension embryos expression is already being down-regulated so that 3-4 cells per abdominal segment now express Atonal (Figure 2.17 C, arrowheads). Shortly after, Atonal expression becomes undetectable in the abdominal segments. While *atonal* is of considerable interest, and is expressed in a subset of peripheral nervous system precursors, it is not expressed in any of the differentiated cells of the chordotonal organs and so will not be of use as a marker of sensory neurons.

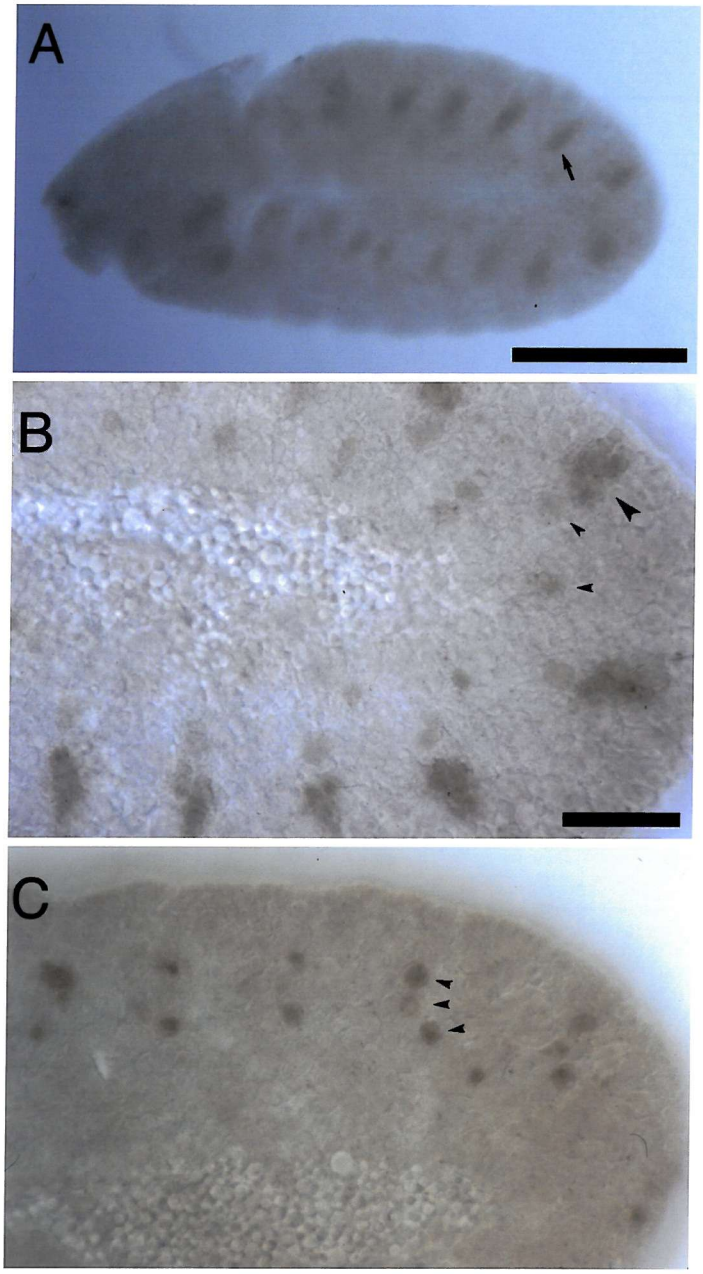


Figure 2.17 Atonal expression in the PNS. **A.** Atonal is expressed in small clusters of epidermal cells during early germ band extension (arrow). **B.** Atonal expression has begun to be restricted to single cells in the dorsal region of each segment (small arrowheads) but is still expressed in a larger cluster of cells in the ventral region of the segment (large arrowhead). **C.** Only three cells per segment still express Atonal (arrowheads). (A) Scale bar 0.1mm. (B-C) Scale bar 0.03mm.

BarH1 and BarH2

BarH1 and *BarH2* are two closely related homeobox genes that are functionally redundant (Higashijima et al., 1992a). Both genes are co-expressed within the embryonic nervous system where they are required for the specification of campaniform-like sensilla fate as opposed to trichoid sensilla fate within the external sense organs (Higashijima et al., 1992). BarH1/H2 expression first begins to be detected during germ band extension (Figure 2.18A) and the complete pattern of BarH1/H2 expression is evident by the completion of germ band shortening (Figure 2.18 B). In addition to their expression within the sensory nervous system BarH1/H2 also label a segmentally repeated stripe of cells in the dorsal region that appear at the boundary of each segment. This epidermal expression may well correspond to the earlier expression detected during germ band extension as expression does not become apparent within the sensory nervous system until the final stages of sense organ development (Higashijima et al., 1992).

Within the sensory nervous system BarH1/H2 expression is confined to the external sense organs, where BarH1/2 have previously been shown to strongly label the glial theocogen cells, with the external sensory neuron itself being more weakly stained (Higashijima et al., 1992). The external sense organs that express BarH1/H2 can be identified by their stereotyped locations within the periphery and by comparison with the location of the chordotonal organs, which although unstained are still visible at the light microscope level (Figure 2.18 D-F). Expression is detectable in the glial thecogen cells of all external sense organs and in all external sensory neurons, although the level of BarH1/H2 expression varies significantly between individual neurons. For example, BarH1/H2 expression is very weak in lesC and lesA, both of which form trichoid sensilla type external sensory organs (Dambly-Chaudiere and Ghysen, 1986) compared to lesB, which forms a campaniform-like sensilla (Dambly-Chaudiere and Ghysen, 1986). It is therefore thought that the level of BarH1/H2 expression determines subtype specification in external sensory organs (Higashijima et al., 1992). One notable exception is the low level of BarH1/H2 protein expression observed in desD, which has previously been classified as a campaniform-like sensilla (Dambly-Chaudiere and Ghysen, 1986). Re-examination of the external morphology of desD suggests that this sensilla may in fact be intermediate between trichoid and campaniform sensilla (Higashijima et al., 1992)

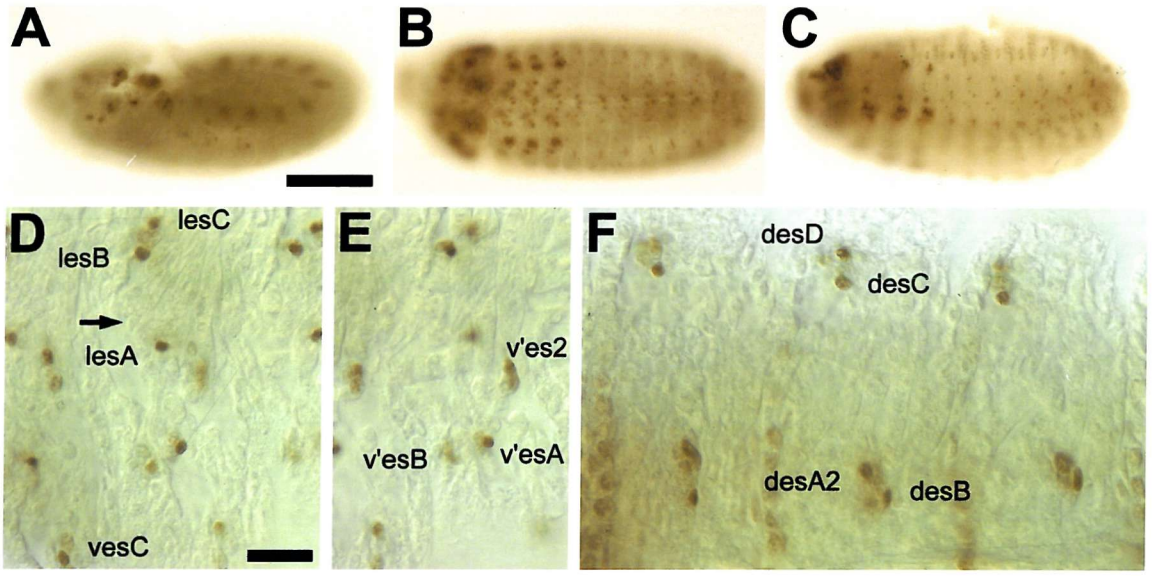


Figure 2.18 BarH1/H2 expression in the PNS. A. BarH1/H2 expression during germ band extension. **B.** Ventral view of BarH1/H2 expression in a stage 15 embryo. **C.** Lateral view of BarH1/H2 expression in a stage 15 embryo. **D, E.** BarH1/H2 expression in the ventral and lateral region. Arrow indicates the position of lch5. Each es organ is labelled and in each case, the most strongly stained cell corresponds to the thecogen cell. **F.** BarH1/H2 expression in the dorsal region. Each es organ is labelled and again, the most strongly stained cell corresponds to the thecogen cell. (A-C) Scale bar 0.1mm. (D-F) Scale bar 0.01mm.

Cut

Cut is required for the formation of external sensory organs (Blochlinger et al., 1990) and is expressed in a subset of cells within the PNS. Cut staining is first detected during germ band extension (data not shown). At this stage Cut labels a small number of peripheral cells in each abdominal segment and these cells have previously been determined to be external sense organ precursors (Blochlinger et al., 1990). The number of Cut-positive cells in each segment progressively expands as development proceeds until the complete pattern of Cut expression within the sensory nervous system is achieved by the end of germ band shortening (Figure 2.19 A). Cut is expressed in all support cells and neurons of the external sensory organs and also labels a number of multidendritic neurons (Figure 2.19 B, C). The Cut-positive multidendritic neurons comprise all four ventral cluster mds, vbd, v'ada, v'pda, ldaA, ldaB, and three of the dorsal cluster mds. Several other cells are labelled in the periphery, and some of these have previously been identified as presumptive glial cells (Blochlinger et al., 1990).

Pox-neuro

Pox-neuro (*pox-n*) encodes a transcription factor that specifies the formation of poly-innervated external sense organs as opposed to mono-innervated external sense organs. As there are only two poly-innervated external sense (p-es) organs, v'esA2 and d'esA2, in each abdominal hemisegment of the embryo, both of which are specified by *pox-n*, it was expected that Pox-n would specifically label only those p-es neurons. The results of the antibody staining were therefore somewhat surprising. Firstly, a stripe of epidermal expression, reminiscent of a segment polarity gene expression pattern, was clearly visible in each segment (Figure 2.20 A). As this expression faded a number of peripheral cells remained clearly labelled (Figure 2.20 C-G). The number and location of these cells was not consistent with Pox-n exclusively labelling p-es neurons. Pox-n also labelled the dendrites of the chordotonal neurons (Figure 2.20 D, arrow). The staining of the chordotonal neuron dendrites clearly cannot truly reflect Pox-n expression as Pox-n is a transcription factor whose expression is confined to the nucleus, and must be artefactual in nature. In addition, as *pox-n* is a paired box (pox) gene it shares regions of sequence homology with the segment polarity gene paired (Dambly-Chaudiere et al., 1992). The epidermal expression observed could perhaps therefore reflect a cross-reaction with the paired protein.

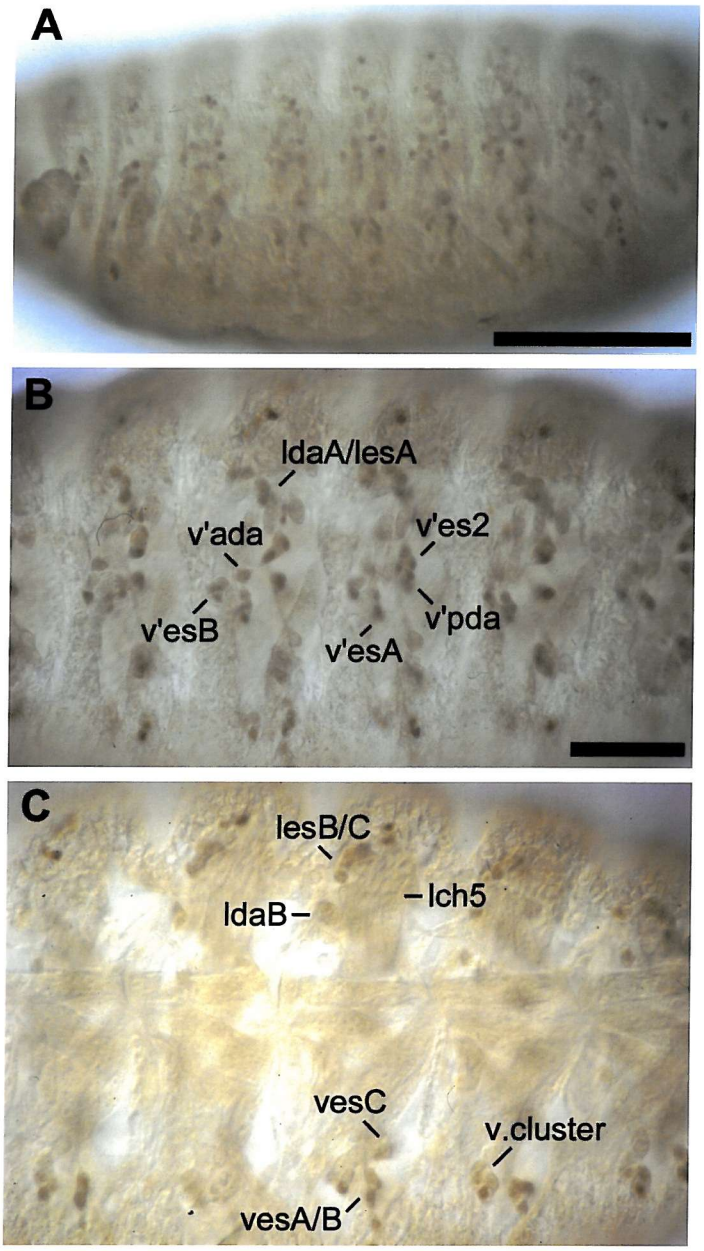
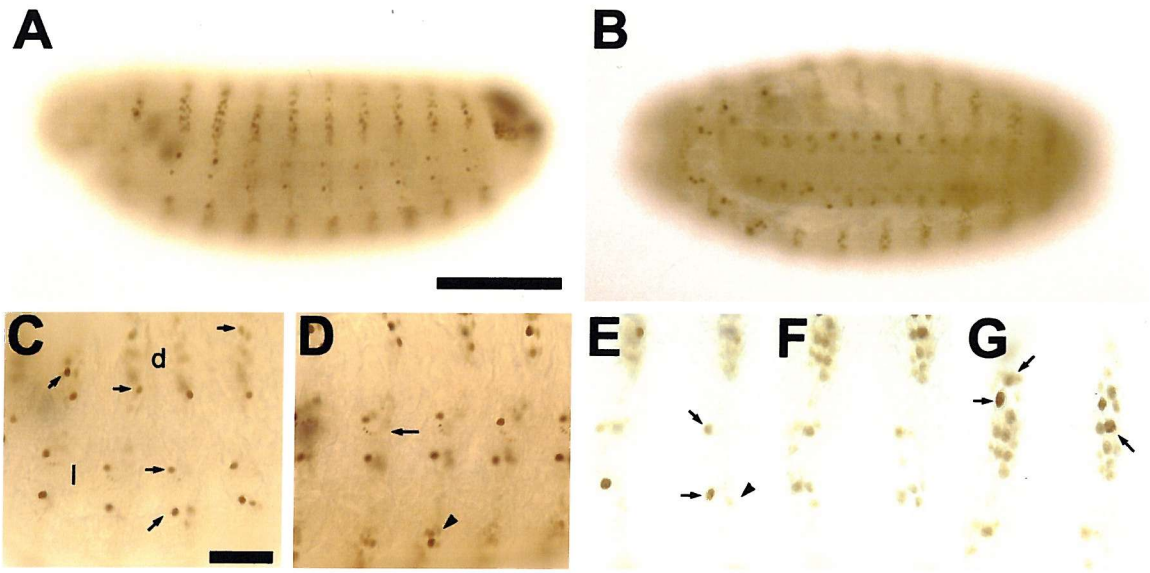


Figure 2.19 Cut expression in the PNS. A. Lateral view showing Cut expression in the periphery. **B.** Cut expression in the ventral and lateral regions of the embryo. **C.** Slightly different plane of focus showing Cut expression in additional ventral and lateral neurons. (A) Scale bar 0.1mm.(B-C) Scale bar 0.03mm.

Figure 2.20 Pox-neuro expression in the PNS. A. Lateral view of Pox-neuro (Pox-n) expression in the periphery. **B.** Ventral view showing Pox-n expression in the ventral nerve cord. **C.** Pox-n is expressed in a discrete number of cells in the lateral and dorsal region (arrows). **D.** Pox-n labels the scolopales of the lch5 neurons (arrow). Arrowhead indicates Pox-n expression in a subset of CNS cells. **E.** Shows Pox-n expression in the lateral region in greater detail. Two cells are stongly labelled (arrows) and expression is also visible in third cell, which lies just out of the plane of focus. **F.** Shows Pox-n expression in the lateral region in a slightly different plane of focus. **G.** Shows Pox-n expression in the dorsal region in greater detail. A number of cells are strongly labelled (arrows). (A-B) Scale bar. 0.1mm. (C-G) Scale bar 0.03mm.

Figure 2.21 Fluorescent detection of Pox-n/Mab22c10 expression in the PNS. Pox-n staining is green. Mab22c10 staining is red. **A.** In the dorsal region Pox-n is expressed in a stripe of epidermal cells that overlie the dorsal cluster of sensory neurons. **B.** Pox-n is expressed in dorsal cluster es neurons. **C.** In the ventral region Pox-n labels two of the lateral es neurons and a third, non-neuronal cell (arrow). **D.** Shows Pox-n/Mab22c10 expression in the lateral region in a slightly different plane of focus. (A-C) Scale bar 15μm.


A
B
C
d
l
D
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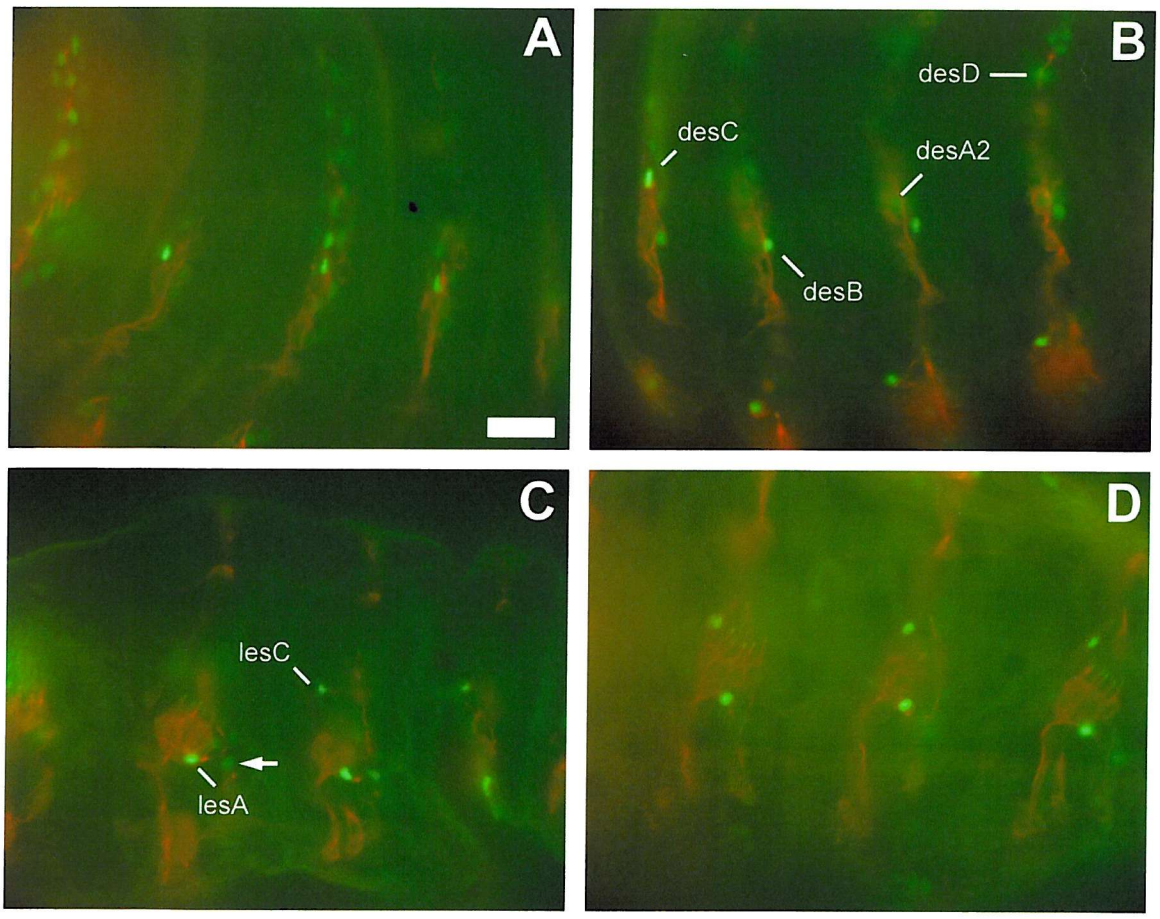

A
B
desD
desC
desA2
desB
C
lesC
lesA
D

To determine the exact identity of the sensory neurons labelled by the Pox-n antibody, embryos were double-stained for Pox-n and Mab22c10. Pox-n expression, as revealed by antibody staining, is observed in a small subset of external sensory neurons. Two sensory neurons are labelled in the lateral region and can be identified as lesA and lesC (Figure 2.21 C, D). A third, more faintly stained cell is also labelled that is level with, and slightly posterior to lesA (Figure 2.21 C, arrow) but is not a sensory neuron. Dorsally the Pox-n antibody labels a number of sensory neurons within the dorsal cluster (Figure 2.21 A, B). Expression is detectable in desB, on the posterior side of the dorsal cluster, desC and desD at the dorsal end of the dorsal cluster and also appears to be faintly present in desA2. A number of epidermal cells overlying the dorsal cluster still show Pox-n expression (Figure 2.21 A). No cells ventral to lesA within the sensory nervous system are detected with the Pox-n antibody therefore this antibody cannot label the ventral poly-innervated external sensory neuron, v'es2.

2.4.3 Expression pattern of potential downstream genes, including cell-surface molecules

Segmentation genes primarily encode transcription factors, and if any of the candidate genes are found to influence sensory axon growth in the CNS, they are likely to act more like master control genes which exert their effect indirectly through the modulation of downstream cell surface molecules. I have therefore also examined the expression of a number of other proteins, such as cell adhesion molecules, which could provide potential targets for segmentation genes such as *engrailed* and *runt*. Many of these genes, such as the *fasciclins*, are already known to play a role in axon guidance in motorneurons and two of these genes, *neurotactin* and *connectin*, are already known to be negatively regulated by *engrailed* in the CNS (Siegler and Jia, 1999). Genes known to be expressed in the optic sensory system, such as *glass* and *rough*, have also been examined in addition to a number of other genes which have previously been described as being expressed in the sensory nervous system.

Table 2.3. Potential downstream genes

Function	Gene	Potential expression in sensory nervous system?
Cell adhesion molecules	*connectin*	No
	fasc I	?
	fascII	No
	fascIII	?
	Neurotactin	Yes
	neuroglian	Yes
	futsch (22c10)	Yes
	ILM-P2	Yes
	chaoptin	No
Transcription factors	*hindsight*	Yes
	elav	Yes
	peanut	No
Secreted proteins	*scabrous*	Yes
	glass	No
	rough	No

While the protein expression pattern was successfully determined for the majority of the genes examined in this group, the expression patterns of Fasciclin I and Fasciclin III in the sensory nervous system have not yet been determined. Despite experimenting with a wide range of antibody dilutions and devittelinising embryos by hand, the staining achieved with the anti-Fasciclin I antibody was insufficient to enable its expression pattern in the PNS to be determined. Fasciclin III is expressed throughout the epidermis and this staining obscures the sensory nervous system, preventing the identification of potential sensory nervous system expression (data not shown). The staining of all other antibodies in this group that showed expression in sensory neurons will now be described.

2.4.3.1 Antibodies that label all sensory neurons

Several of the genes included in this analysis are expressed in all sensory neurons. *Neuroglian*, *futsch*, *elav*, and *scabrous* were known to be expressed in all sensory neurons and during the course of this investigation another gene, *hindsight*, was also discovered to label all sensory neurons. Such genes can provide useful markers for the sensory nervous system. *Elav* and *futsch* in particular have proven invaluable in determining the expression pattern of many of the other genes investigated as they label distinct cellular compartments. *Elav* encodes an RNA binding protein that regulates the differential splicing of a number of target genes, including *neuroglian*, and labels the nuclei of all neurons (Figure 2.22 B, D). The recently identified *futsch* gene (Hummel et al., 2000) encodes the antigen recognised by Mab22c10, which stains the cytoplasm, dendrites and axons of all embryonic sensory neurons and a subset of central neurons (Figure 2.22 A, C) and has been widely used to visualise neuronal morphology. The Elav and Futsch proteins therefore provide complementary markers for the entire sensory nervous system.

Of the other antibodies showing pan-neural expression the anti-Neuroglian antibody, which recognises a neuronal splice-variant of the cell adhesion molecule Neuroglian, labels the cell membrane of all sensory neurons. The Scabrous antibody reveals a punctate staining pattern with the antigen appearing to accumulate in neighbouring non-neuronal cells, probably reflecting the fact that scabrous is a secreted molecule. Hindsight labels the nuclei of all PNS neurons and associated cells, as well as a large number of cells within the amnioserosa.

2.4.3.2 Antibodies that label a subset of sensory neurons

Fasciclin II

Fasciclin II encodes an immunoglobulin superfamily cell adhesion molecule (IgCAM) which is expressed by motorneurons and a subset of CNS neurons (Grenningloh et al., 1991). This is shown in Figure 2.23 A and B. Fasciclin II also stains a single sensory neuron, lbd (Figure 2.23 C, D). The axon, dendrites and cell body of lbd are revealed by Fasciclin II expression. No other sensory neurons appear to express Fasciclin II although it is possible that Fasciclin II expression on the axons of sensory neurons might not be detected where sensory and motor axons fasciculate together within the segmental (SN) and intersegmental nerves (ISN). Fasciclin II labels the cell body of lbd however and no other sensory neurons cell bodies

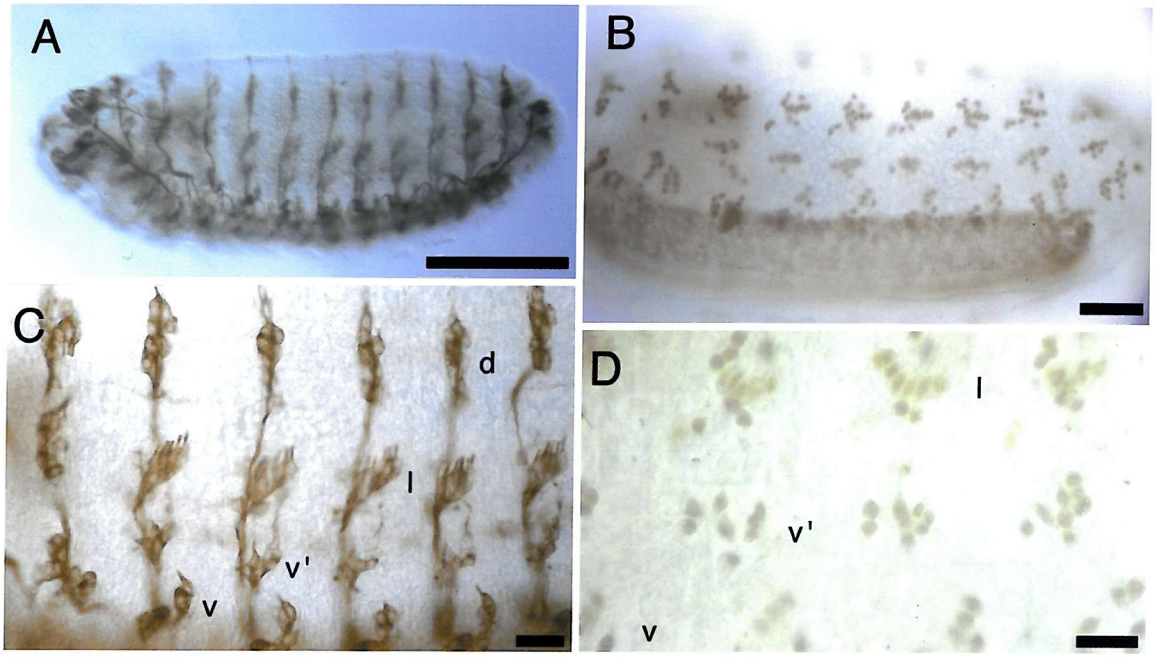


Figure 2.22 Antibodies that label all PNS neurons. A, C. Mab22c10 expression. B, D. Elav expression. **A.** Lateral view of Mab22c10 expression in the PNS. **B.** Lateral view of Elav expression in the PNS **C.** Mab22c10 labels the membranes and axons of all PNS cells. **D.** Elav labels the nuclei of all PNS cells. v - ventral, v' - ventro-lateral, l - lateral, d - dorsal. (A) Scale bar 0.1mm. (B) Scale bar 0.03mm. (C) Scale bar 0.01mm. (D) Scale bar 0.01mm.

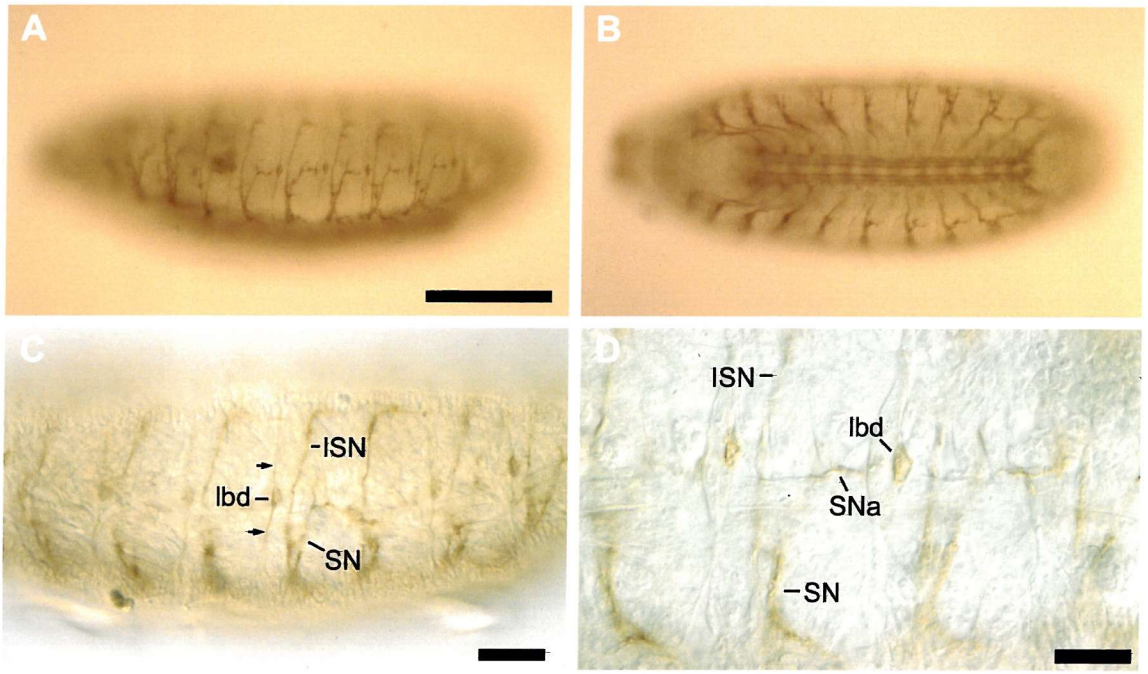


Figure 2.23 Fasciclin II expression in the PNS. A. Lateral view showing peripheral expression. **B.** Ventral view showing CNS expression. Fasciclin II labels a subset of CNS axon pathways. **C.** In the periphery the axons of motorneurons in the peripheral nerves and the cell body and dendrites (arrows) of lbd are stained. **D.** Shows the peripheral expression pattern in greater detail. (A-B) Scale bar 0.1mm. (C) Scale bar 0.03mm. (D) Scale bar 15μm.

show Fasciclin II expression, suggesting that Fasciclin II expression in the sensory nervous system is restricted to lbd.

IMP-L2

The IMP-L2 protein has been described as exclusively labelling lbd within the sensory nervous system (Garbe et al., 1993). Although the IMP-L2 antibody was obtained, it had not been used for several years and it was not known whether it was still functional (J. Natzle, personal communication). Weak embryonic staining was achieved, which did label lbd (Figure 2.24 B, C), however to improve the quality of the staining the antibody will need to be affinity-purified.

Neurotactin

Neurotactin is a transmembrane protein that functions in cell adhesion. Neurotactin stains a subset of cells within the sensory nervous system. As Neurotactin is localised to the membrane of expressing cells, the morphology of each sensory neuron that expresses neurotactin is revealed, enabling their identity to be determined. Neurotactin is expressed in all chordotonal organs, where both the chordotonal neurons and the soma sheath support cells are labelled (Figure 2.25 D). Neurotactin is also expressed in all external sense organs, where again both the external sensory neurons and a number of support cells are labelled (Figure 2.25 E, F, G). The exact identity of the support cells that express neurotactin in external sense organs has not been determined though from their positions it would appear that the soma sheath cell is again labelled in most or all external sense organs. A second cell is also labelled distal to the external sensory neuron. External sensory neuron dendrites terminate at or within this cell, which must correspond to either the tricogen or the tormogen. Neurotactin also labels the dbd support cell (Figure 2.25 G) although there does not appear to be expression within dbd itself. Of the multidendritic neurons only dbd is known to have a support cell. No multidendritic neurons themselves appear to express Neurotactin.

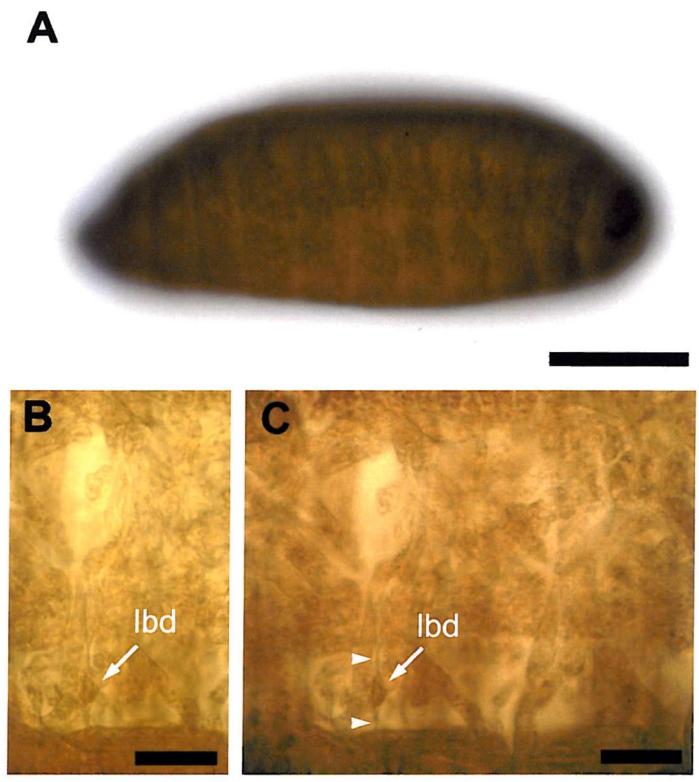


Figure 2.24 IMP-L2 expression in the PNS. A. Lateral view showing peripheral expression. **B.** Lbd is faintly stained. The dendrites of lbd can be seen extending dorsally and ventrally along muscle 8 (arrowheads). **C.** Shows the faint staining of lbd (arrow) in greater detail. (A) Scale bar 0.1mm. (B-C) Scale bar 15μm.

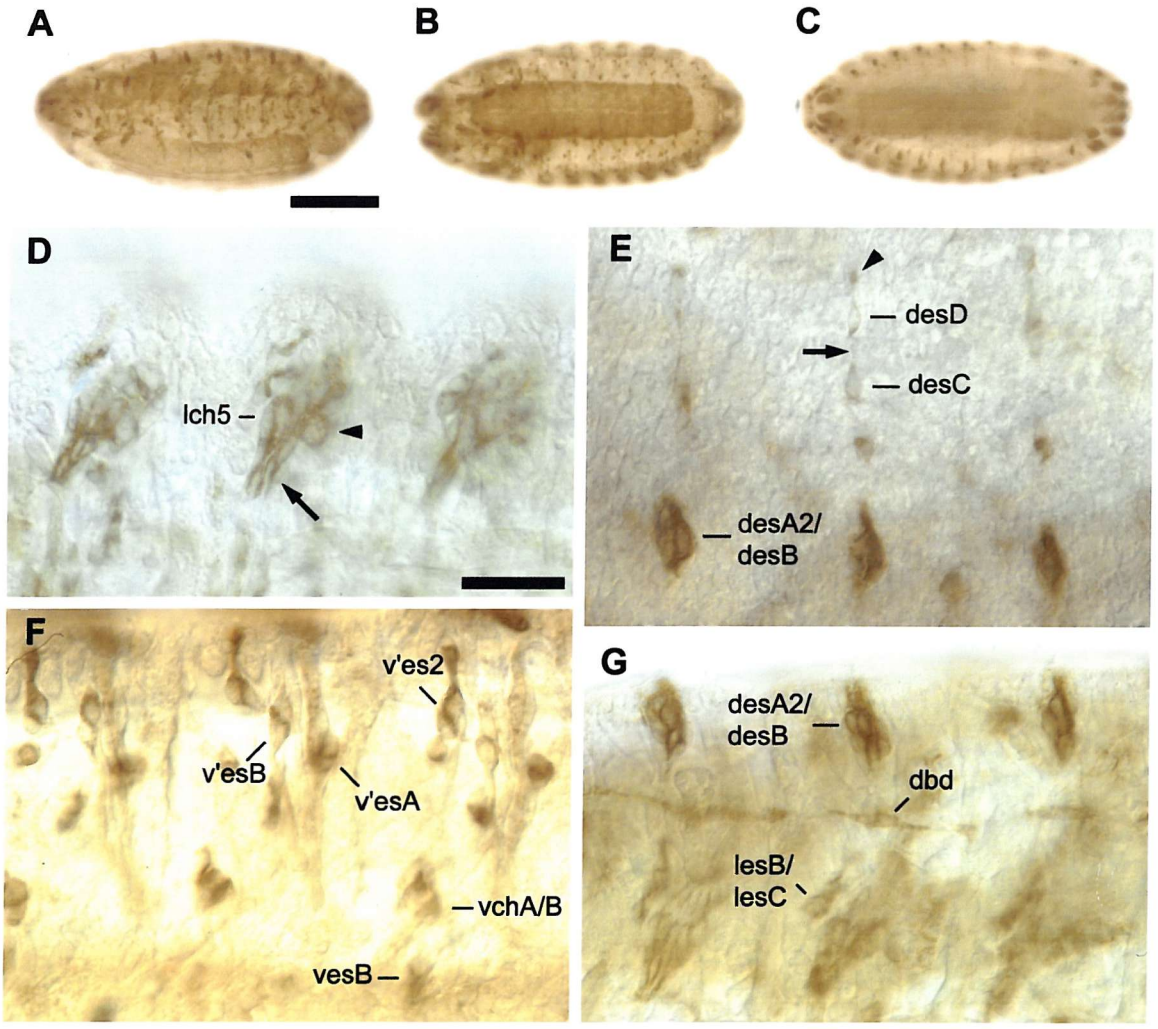


Figure 2.25 Neurotactin expression in the PNS. A. Lateral view showing Neurotactin expression in the periphery. **B.** Ventral view showing Neurotactin expression in the ventral nerve cord. **C.** Dorsal view of Neurotactin peripheral expression. **D.** Neurotactin is expressed in lch5 neurons (arrowhead) and support cells (arrow). **E.** Neurotactin is expressed in dorsal es neurons and support cells. Arrowhead indicates desD dendrite and arrow indicates desC dendrite. **F.** Neurotactin is expressed in all ventral and ventro-lateral es organs and vchA and vchB. **G.** Neurotactin also labels the dbd support cell. (A-C) Scale bar 0.1mm. (D-G) Scale bar 0.01mm.

2.5 Discussion

I have used a combination of antibodies and enhancer-trap lines that are expressed in the pattern of specific genes to examine the expression patterns of a wide number of genes within the sensory nervous system. This has enabled me to create a map of gene expression within each sensory neuron that is summarised in Figure 2.26. Such a map of gene expression enables the majority of sensory neurons to be identified by the unique combination of genes that they express and provides a useful tool for examining the mechanisms that determine cell fate in the sensory nervous system, both in the identification of candidate genes that may be involved in specifying cell fate and in providing molecular cell fate markers.

The patterns of gene expression within differentiated sensory neurons are shown in Figure 2.26 as, with the exception of *atonal* and *cut* which were already known to label sense organ precursors, none of the genes tested showed expression within the sensory nervous system at earlier stages in its development. Each gene labels a precise subset of sensory neurons and one of the unexpected findings from this study is that many of these genes label a very small number of sensory neurons, or even just a single neuron (*IMP-L2*). The expression of these genes in such restricted patterns of sensory neurons suggests that gene expression is tightly regulated within the sensory nervous system and that the expression of at least some of these genes may contribute to cell fate determination in the sensory nervous system. Furthermore, the fact that not all neurons of the same modality express the same combination of genes suggests that these neurons, while apparently identical in outward appearance, may not all adopt the same fate within the sensory nervous system.

2.5.1 Segmentation gene expression in the sensory nervous system

Several segmentation genes are re-expressed during PNS development and do show restricted patterns of expression within the sensory nervous system. *Runt*, *armadillo*, and *engrailed* are all expressed in discrete subsets of sensory neurons while *sloppy-paired* is likely to be expressed in peripheral glia associated with the sensory nervous system. The huckebein-lacZ enhancer-trap line also labels a subset of sensory neurons, although this does not necessarily indicate that *huckebein* itself is expressed in sensory neurons. With the exception of *armadillo*, which is expressed in all chordotonal and external sensory neurons, the segmentation genes are expressed in very small subsets of sensory neurons, with each antibody labelling between 3 and 5 sensory neurons per hemisegment.

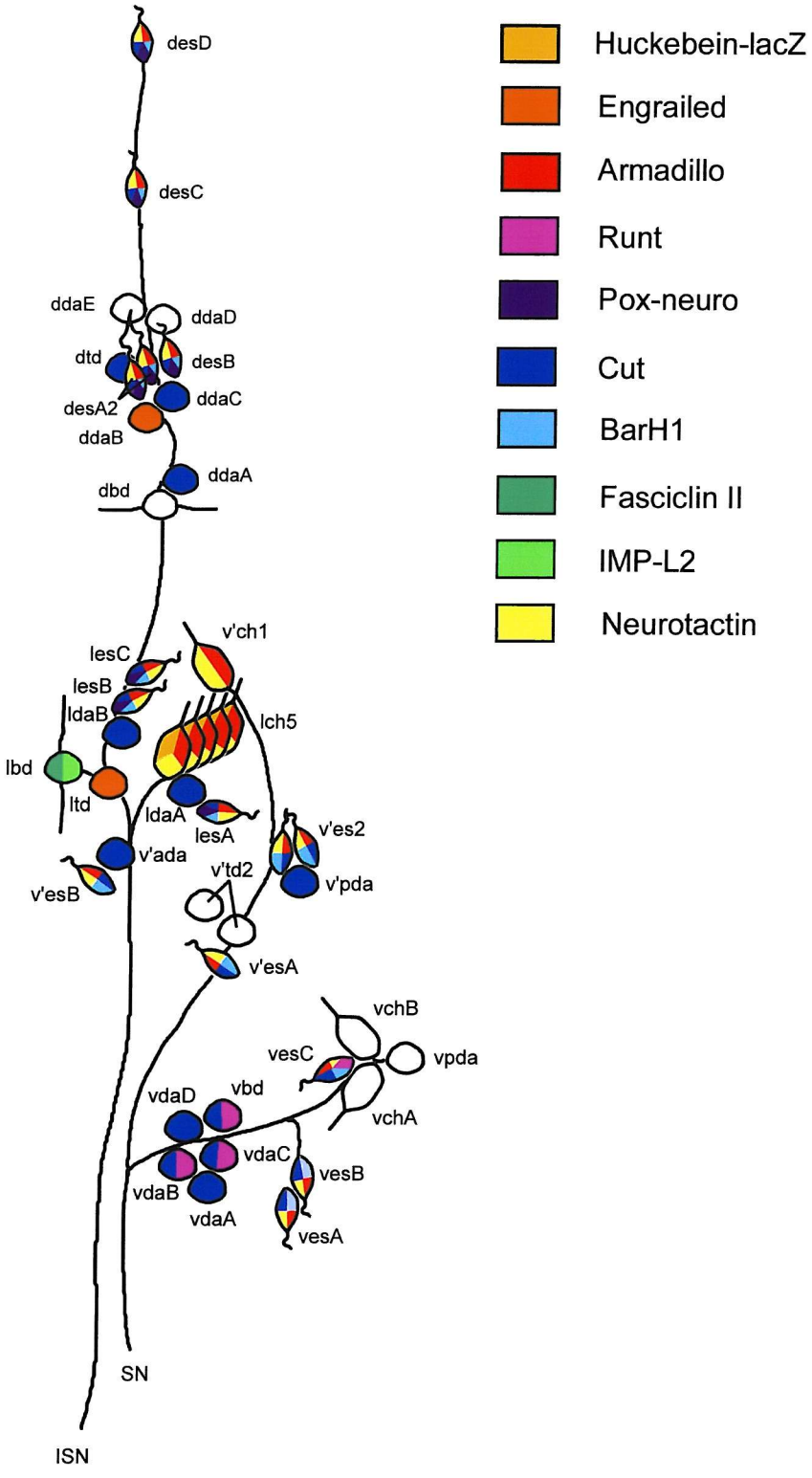


Figure 2.26 Map of gene expression in the sensory nervous system. Summary of the patterns of expression observed within the embryonic sensory nervous system.

The fact that so few neurons are labelled by the expression of segmentation genes and that each gene is expressed in such a small subset of cells may imply that certain cells may play a greater role than others in the generation of an ordered nervous system. During nervous system development a small number of neurons are thought to function as pioneer neurons and establish the major nerve routes in the peripheral and central nervous systems which guide the growth of later born neurons (Bate, 1976; Goodman et al., 1984; Goodman et al., 1981; Ho and Goodman, 1982; Keshishian, 1980; Bentley and Keshishian, 1982; Keshishian and Bentley, 1983b; Keshishian and Bentley, 1983c). While the exact requirement for pioneer neurons remains unclear, it has recently been shown that persistent larval sensory neurons guide the assembly of ordered sensory projections in the adult (Williams and Shepherd, 2002). This suggests that the ordering of the sensory nervous system is accomplished by establishing an ordered array of sensory afferents in the smaller, less complex environment of the embryonic CNS.

The major sensory axon pathways within the adult CNS might therefore be pioneered by a small number of persistent embryonic sensory neurons. Similarly, the segregation of sensory afferents within the embryonic CNS could also be accomplished through the use of a small number of sensory neurons whose axonal projections provide a framework upon which the elaboration of orderly arrays of sensory afferents can occur. Perhaps the expression of the segmentation genes is restricted to a select few sensory neurons because these are the neurons that guide the formation of the embryonic pattern of central projections. Rather than providing positional information to each individual sensory neuron, segmentation genes might therefore only specify positional values within these pivotal sensory neurons. It would be interesting to determine whether the neurons that express segmentation gene products establish their patterns of central projections earlier than their unlabelled counterparts and whether ablation of such neurons affects the establishment of an ordered pattern of central projections within the embryonic nervous system.

It is also conceivable that the same group of sensory neurons could fulfil guidance functions in both the embryonic and adult sensory systems. This scenario is not consistent with such a role being executed by the segmentation gene expressing sensory neurons however, as almost all of these neurons do not survive metamorphosis. The only persistent sensory neurons to be labelled by segmentation gene expression are in fact the *engrailed* expressing multidendritic neurons ltd and ddaB.

2.5.2 Is positional information likely to be encoded by re-expression of segmentation genes?

As discussed above, the fact that so few neurons have been identified to express segmentation genes could imply that a small number of neurons may play a greater role in establishing an ordered array of sensory projections. While each of the major peripheral domains is represented, would such a small number of cells be sufficient to establish positional values within the nervous system? Additional segmentation genes are still being identified (Baumgartner et al., 1994; Epps et al., 1997; Tang et al., 2001), and it is possible that further sensory neurons could express a number of these novel segmentation genes. Alternatively, while segmentation gene expression within specific sensory neurons may confer some aspect of sensory neuron identity, such expression may not be providing positional information within that neuron. Indeed, the sensory neurons in which a given segmentation gene is expressed bear little relation to the positional values defined by the earlier expression of that gene, arguing against a role in positional determination. For example, *engrailed* expression as a segment polarity gene defines the posterior region of each segment yet *engrailed* is expressed within the PNS in the lateral tracheal dendritic neuron, ltd, one of the most anterior neurons in each abdominal segment and furthermore a neuron which does not emerge from the *engrailed* stripe.

2.5.3 Is positional information encoded during the initial process of patterning the embryo?

As discussed previously, studies in other insects indicate that it is likely that positional information is somehow encoded during the differentiation of sensory neurons, and that positional information in the periphery determines target region within the CNS (Murphey et al., 1980; Murphey et al., 1983a; Murphey et al., 1989b; Walthall and Murphey, 1984). SOPs delaminate in a dorsal-to-ventral wave during the period of segment polarity gene activity and it is possible that, rather than segmentation genes being re-expressed during sensory nervous system development, the positional information already present in the epidermis during the period of SOP delamination is stably inherited by the SOPs.

In a wide range of species epigenetic inheritance is accomplished by differential DNA methylation but *Drosophila* does not methylate its genome. One mechanism by which the stable transmission of epigenetic information, such as positional information, could be accomplished in *Drosophila* however is via the Polycomb and trithorax group proteins. These

proteins function to preserve transcription patterns throughout development and can modify the local chromatin environment to either promote gene transcription or to silence target gene expression (Ringrose and Paro, 2001). Polycomb and trithorax group proteins bind to regulatory sequences known as Polycomb Response Elements and maintain transcriptional repression or transcriptional activation, respectively. The Polycomb group proteins have recently been shown to form large multiprotein complexes with the general transcriptional machinery, suggesting they may regulate transcription through direct physical contact with the general transcriptional machinery (Breiling et al., 2001; Saurin et al., 2001). Known targets include the homeotic genes and, interestingly, *engrailed* (Orlando et al., 1998; Strutt et al., 1997; Strutt and Paro, 1997). Activation of Polycomb and trithorax group proteins could therefore ensure that positional information present in the epidermis during SOP delamination was stably transmitted to the sensory neuron progeny of each SOP.

2.5.4 Armadillo expression within the sensory nervous system

Armadillo is expressed by all mechanosensory neurons, where it is localised to the dendrites of external sensory neurons and chordotonal neurons. The expression of Armadillo is thus restricted according to sensory modality but occurs throughout the sensory nervous system and is therefore unlikely to be providing positional information within sensory neurons. As *armadillo*, the *Drosophila* homologue of *β-catenin*, is required for the formation of adherens junctions, it is possible that the staining observed reflects *armadillo's* role in junction formation rather than an instructive role for *armadillo* in the development of the sensory nervous system. Adherens junctions are normally found at the apical end of the lateral membrane between epithelial cells, where they maintain cell adhesion. Armadillo accumulates in sensory neurons at the dendrite, which is located at the dorsal end of the cell. Adherens junctions occupy a more lateral position in epidermal cells but this discrepancy in the location of Armadillo staining could be explained by the fact that sense organ precursors delaminate from the epidermis and sensory neurons are not therefore surrounded by epithelial cell neighbours. Sensory neurons would not necessarily need to form junctions with neighbouring cells but may well need to form junctions with the epidermally-derived cells that are part of the same sense organ and form part of the signal transducing apparatus. In the case of the external sensory neurons, the dendrite innervates the hair cell which will form the cuticular sense organ while the chordotonal neuron dendrite innervates the scolopale. In order to transduce the sensory signal, strong cell adhesion between the support cell and neuron may well be required. As Armadillo is part of a protein complex that connects the adherens junction to the cytoskeleton, such an association could provide contractile function within the

neuron. A role for Armadillo in mediating cell adhesion between sensory neurons and their associated support cells would also provide an explanation as to why Armadillo expression was only observed in type I sensory neurons. Alternatively, perhaps the expression of Armadillo within the nervous system reflects the fact that *armadillo* is required for cells to maintain cellular polarity, presumably through its association with the adherens junction. The bipolar mechanosensory neurons may therefore require *armadillo* to maintain their polarity whereas the multipolar multidendritic neurons do not necessarily maintain apical-basal polarity.

2.5.5 *Engrailed* is a prime candidate for controlling axonal projections

Engrailed is transiently expressed in three or four out of the five chordotonal neuron of the lateral pentascolopale (this expression will be discussed in relation to the development of the chordotonal organs in the next section) but is also expressed in a single neuron of the dorsal cluster, ddaB and the lateral tracheal dendritic neuron ltd. That Engrailed is maintained in just two cells per hemisegment is particularly intriguing. As Engrailed is downregulated in other neurons and in the epidermis at this time, the active maintenance of Engrailed expression within these two cells may influence the pattern of development of that cell. It is also of note that both Engrailed-expressing sensory neurons persist throughout metamorphosis and, as persistent sensory neurons guide the central projections of the adult complement of sensory neurons (Williams and Shepherd, 2002), these neurons could possibly also play a pivotal role in the development of the adult sensory nervous system. This raises the possibility that these neurons persist because they express *engrailed* and that expression of *engrailed* could be required for these neurons to persist throughout metamorphosis, an idea that can be tested by examining the effects of *engrailed* loss of function and ectopic expression on the fate of sensory neurons.

There is considerable evidence from both insects and vertebrates to support the idea that expression of *engrailed* could control axonal projections. Differential expression of *engrailed* regulates axonal patterning in the tectum of a number of vertebrates, including chicks, mice and Xenopus (Friedman and Oleary, 1996; Logan et al., 1996; Retaux et al., 1996). In the cercus of the cockroach, *Periplanta americana*, *engrailed* is expressed in the medial sensory neurons but not in the lateral sensory neurons and could therefore provide positional information to these neurons (Blagburn et al., 1995). Abolition of *engrailed* expression in the cockroach through injection of double-stranded RNA (RNAi) transformed the axonal projections of a typical *engrailed*-positive neuron from medial type to lateral type and also

resulted in a change in synaptic specificity, with connections characteristic of lateral type neurons being formed (Marie et al., 2000). *Engrailed* cannot be solely responsible for determining axonal projection pattern and synaptic connectivity in the cockroach however, as not all *engrailed*-positive neurons exhibit the same type of axonal morphology or share the same synaptic partners (Blagburn et al., 1995).

As *engrailed* codes for a transcription factor it could potentially act as a master control gene and regulate many aspects of neuronal identity, including the expression of downstream cell-adhesion molecules and cell-surface receptors to determine axon trajectory. The changes resulting from loss of *engrailed* function in the cockroach strongly support the idea that *engrailed* regulates multiple downstream cell-surface targets (Marie et al., 2000). The *connectin* and *neuroglian* cell adhesion molecules have been identified as downstream targets of *engrailed* function in *Drosophila* nervous system development where *engrailed* has been shown to negatively regulate their expression in the embryonic central nervous system (Siegler and Jia, 1999). The regulation of cell adhesion molecules by *engrailed* provides a mechanism by which *engrailed* could influence the neuronal morphology of sensory neurons. *Neuroglian* and *connectin* are unlikely to be targets of *engrailed* transcription in the sensory nervous system however, as *neuroglian* is expressed in all sensory neurons while *connectin* is not expressed in any. In conclusion, *engrailed* is a very strong candidate for controlling axonal projections in the *Drosophila* embryo and has therefore been selected as the prime focus of further experiments.

2.5.6 The development of the chordotonal organs

The formation of the chordotonal organs requires *atonal* function (Jarman et al., 1993), but *atonal* is only expressed in five of the eight chordotonal precursors. Lineage analysis has shown that one precursor forms the v'ch1 chordotonal organ, one forms either vchA or vchB and the remaining three are incorporated into the lateral pentascolopale, lch5 (zurLage et al., 1997). This implies that vchA or B and two lch5 neurons must be specified by a separate *atonal*-dependent mechanism. The formation of the remaining chordotonal organs has been shown to require the activity of genes that participate in the *Drosophila* epidermal growth factor receptor (DER) signalling pathway (Okabe and Okano, 1997; zur Lage et al., 1997). The *atonal*-expressing founder cells would therefore seem to recruit the remaining neural precursors via a local signalling mechanism involving the *Drosophila* DER pathway.

It has recently been demonstrated that *engrailed* expressing cells within the epidermis can respond to either *wingless* or *spitz* (the activating ligand for DER) and that, depending on the relative level of activation resulting from each pathway, these cells will adopt different fates (O'Keefe et al., 1997). DER signalling is known be required in the formation of the chordotonal organs and the initial stripe of *wingless* expression lies next to the stripe of *engrailed* expression. As the chordotonal organs emerge from the *engrailed* stripe and transient *engrailed* expression is seen in at least three of the chordotonal neurons of the pentascolopale, it may be that some form of competitive interaction between *wingless* and DER signalling in *engrailed* expressing cells is also required in the specification of fate within the chordotonal organs. Perhaps *engrailed* is expressed, at some point, in all five neurons of the pentascolopale and that this activity is required for cells to be able to respond to instructive signals from *spitz* and *wingless*. It is also possible that only those neurons resulting from *atonal* specified precursors express *engrailed* and that this expression distinguishes these chordotonal neurons from their companions in some way.

2.5.7 The expression patterns of potential downstream genes.

IMP-L2, Fasciclin II and Neurotactin all showed restricted patterns of expression within the sensory nervous system. As both Fasciclin II and IMP-L2 are members of the immunoglobulin superfamily (Grenningloh et al., 1991; Garbe et al., 1993), and *neurotactin* encodes a transmembrane cell adhesion protein (de la Escalera et al., 1991), all could fulfil downstream roles in axon guidance. Neurotactin is expressed in all type I sensory neurons, and may therefore fulfil a general role in axon guidance and fasciculation for these neurons. It has been suggested that expression of Neurotactin in retinal photoreceptors may mediate their clustering (de la Escalera et al., 1991) and it is possible that Neurotactin could play a similar role in the clustering of the lch5 chordotonal neurons. IMP-L2 and Fasciclin II show an extremely restricted pattern of sensory nervous system expression and are therefore ideal candidates for downstream genes that guides the growth of sensory axons in a neuron-specific manner. None of the other genes identified in this screen show expression in lbd however, so IMP-L2 or Fasciclin II expression cannot be regulated by any of these genes.

Chapter 3. Embryonic enhancer-trap screen

3.1 Aim

The aim of the work presented in this chapter was to identify P[GAL4] enhancer-trap lines whose expression is restricted within the sensory nervous system, enabling ectopic gene expression to be driven in subsets of sensory neurons. This chapter will summarise the patterns of expression that were observed during the screen and enhancer-trap lines containing embryonic sensory neurons will be described in greater detail. The utility of these lines, as well as any general principles to emerge from the screen, will be discussed.

3.2 Introduction

The GAL4 enhancer-trap technique (Brand and Perrimon, 1993) is one of the most widely used tools in *Drosophila* neurobiology. It enables known genes to be ectopically activated in a cell- or tissue-specific manner, and allows for temporal control of that expression. As such it has proved extremely useful for a wide variety of different types of analysis with respect of gene function and cellular mechanisms. It has been used to chemically ablate specific groups of cells (Hidalgo et al., 1995), to disrupt cellular function (Reddy et al., 1997; Sweeney et al., 1995), to study behaviour (Ferveur et al., 1997; O'Dell et al., 1995) and as a neuroanatomical tool to study functional organisation (Shepherd and Smith, 1996; Yang et al., 1995).

Perhaps the primary application of the technique however, is as a means of achieving directed ectopic gene expression. Ectopic expression can be a powerful way to elucidate the role of a gene in determining cellular identity during development. For example, whereas loss-of-function mutations showed that *eyeless* is required for eye development, ectopic expression using the GAL4 system induced the formation of additional eye structures, indicating *eyeless* is likely to be the master control gene for eye morphogeneis (Halder et al., 1995). The system can also be used to generate loss of function phenotypes by driving the expression of ds-RNA and dominant negative forms of a gene in specific cell types or at specific times in development. This enables problems of early lethality resulting from loss-of-function to be overcome, or a possible requirement for a gene product at several stages during the development of a single structure to be examined (Freeman, 1996).

Essentially the technique involves crossing two lines of transgenic flies: one line carries the yeast GAL4 transcriptional activator under the control of an endogenous promoter or

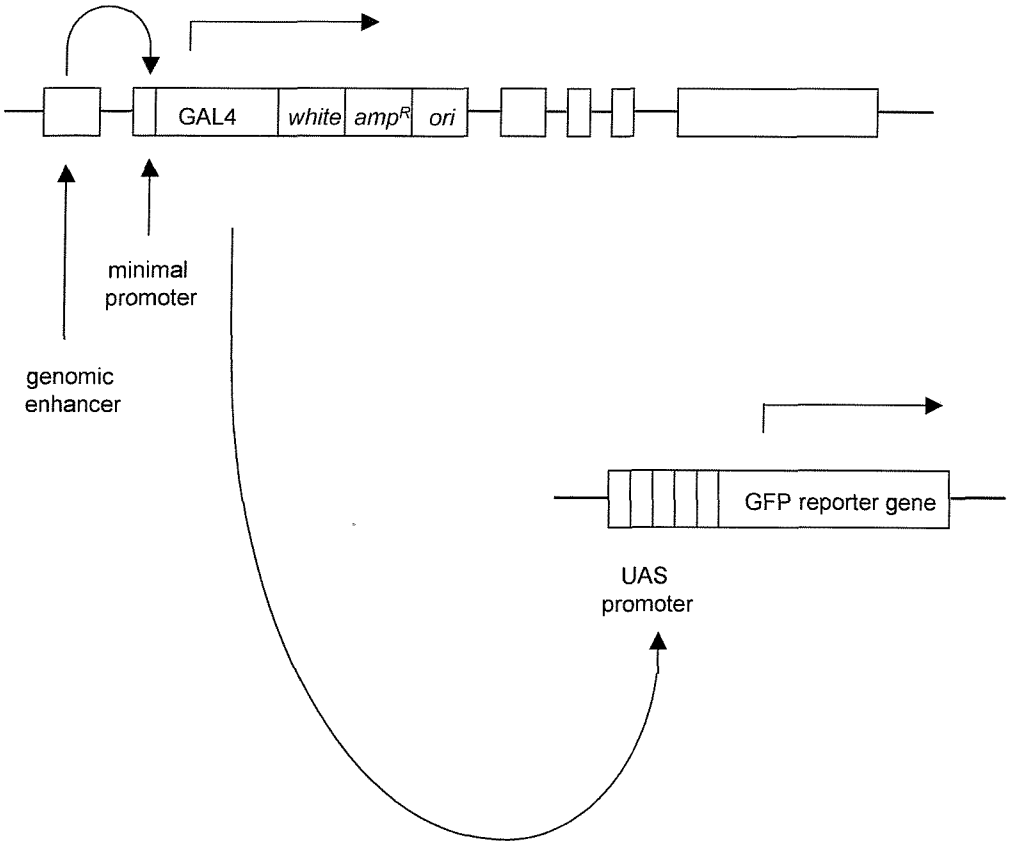


Figure 3.1 The GAL4 Enhancer-trap Technique. P element vectors can be engineered to contain a reporter gene with a very weak promoter. The resulting expression of the reporter gene in a transgenic fly will be so low that its activity will not normally be detected during assays. However, if the construct inserts into the genome in close proximity to an enhancer element, the reporter gene will be expressed in the same temporal and spatial pattern as the active enhancer. The presence of marker genes such as *white* on the construct enable the detection of flies carrying insertions. The ampicillin resistance marker (*amp*^{R}) and the E. coli origin of replication *(ori)* allow plasmid rescue of the sequences flanking the inserts. The yeast transcription factor GAL4 is used as the reporter gene for the enhancer-trap element. A second construct composed of the yeast GAL4-responsive element UAS coupled to a secondary reporter gene such as GFP remains untranslated until crossed to a line of flies containing the GAL4 construct. Transcriptional activation of the UAS-GFP construct by GAL4 produces a pattern of GFP activity that reflects GAL4 activity under enhancer control.

enhancer which directs GAL4 expression in a distinct spatial or temporal pattern; the other contains the coding sequence for the gene of interest under the control of the GAL4 target promoter, the upstream activating sequences (UAS). This is shown in Figure 3.1. As the GAL4 system is derived from the yeast, *Saccheromyces cerevisciae*, it is not endogenously expressed in *Drosophila* therefore, in the absence of the GAL4 activator, the target gene remains silent. Crossing both lines generates progeny that express the gene of interest in the same temporal and spatial pattern in which GAL4 is being expressed. This enables stable transgenic lines to be established, even if the target gene is toxic or lethal. The system therefore provides a convenient means of selectively misexpressing a wide variety of different genes, simply by crossing a suitable GAL4 line with each different UAS-target gene line to be tested.

In the peripheral nervous system, many genes are expressed in subsets of neurons but their function in those cells is unknown. Ectopic expression of these genes in different subsets of neurons could help elucidate their function within the nervous system. The GAL4 system provides a convenient means of misexpressing a variety of different genes in discrete subset of neurons, as almost all of the genes identified as being of interest from the antibody screen which was undertaken in the previous chapter are already available as UAS-lines. Before this can be accomplished however, suitable P[GAL4] enhancer-trap lines to be used as misexpression vectors need to be identified. As this was a minor aspect of the overall project, rather than initiating a new screen to isolate suitable P[GAL4] lines an existing collection of enhancer-trap lines that label sensory neurons at later stages of development was screened, in order to find lines that begin to be expressed in subsets of embryonic sensory neurons at early stages of neurogenesis or prior to axon outgrowth. Such lines would enable a gene to be misexpressed throughout the development of that neuron. The effect of that gene on the axon pathfinding and central projections of a given neuron can then be tested.

3.3 Materials and Methods

3.3.1 Fly Stocks

The P[GAL4] enhancer-trap collection to be screened was generated in the laboratory of Professor Kim Kaiser (University of Glasgow). All flystocks were maintained on standard Drosophila media (Appendix A) at room temperature. Enhancer-trap expression patterns were visualised by crossing males of each P[GAL4]line to be tested to virgin females homozygous for the UAS-tau-GFP reporter gene construct (Andrea Brand, Univeristy of Cambridge).

3.3.2 Screening of P[GAL4] lines

Embryos were collected on apple-juice agar plates (Appendix A). Each cross was left to lay overnight at 25^{0}C to yield embryos at a variety of developmental stages. For initial screening, embryos were manually dechorionated by adhering each embryo to a slide coated with double-sided sellotape and gently popping the embryo out of the chorion using a pair of blunt forceps. Embryos were then transferred to a glue-coated coverslip (double-sided sellotape dissolved in heptane) and covered with 10S Voltalef fluorocarbon oil to prevent desiccation. The coverslip was mounted on a slide using slivers of plasticine to hold the coverslip in place and prevent the embryos from being squashed. The initial screening of embryos was performed using a Zeiss Axiophot II microscope equipped with epifluorescence.

3.3.3 Immunohistochemistry

Immunohistochemistry was carried out as described in Section 2.2 and GFP expression was visualised using an anti-GFP monoclonal antibody mix (Boehringer Mannheim) at a dilution of 1:250.

3.3.4 Microscopy

Stained embryos were examined using a Zeiss Axiophot II microscope. Images were captured on Kodak Ektachrome Elite 160T film using Axiophot software and digitised using a Nikon Coolscan II slide scanner. All figures were created in Adobe Photoshop 5.0 on Apple Macintosh computers.

3.3.5 Staging of embryos

Embryonic staging is according to Nüsslein-Volhard and Wieschaus (Wieschaus and Nüsslein-Volhard, 1998).

3.4. Results

3.4.1. Overview of embryonic P[GAL4] enhancer-trap screen

This screen was based on an existing collection of 42 lines. These lines were originally selected by Smith and Shepherd (Smith and Shepherd, 1996) as part of their screen of 1400 P[GAL4] lines and retained on the basis of expression in subsets of pupal sensory neurons. The expression pattern of each line has previously been examined within the pupal CNS and in the larval PNS and CNS (Smith and Shepherd, 1996; Glossop, 1997). Each P[GAL4] enhancer-trap line was crossed to a line containing the UAS-tau-green fluorescent protein (GFP) reporter gene on the 3rd chromosome. In order to quickly identify lines with potential expression in the embryonic sensory nervous system, each line was preliminarily screened using fluorescent microscopy. Those lines that showed peripheral GFP expression were selected and re-screened using immunohistochemistry to determine the embryonic pattern of expression.

Of the 42 lines in the collection, 27 were examined immunohistochemically after being selected under epifluorescence and 21 of these lines showed expression in the embryonic PNS. Of the P[GAL4] enhancer-trap lines that do show expression within the embryonic sensory nervous system, very few label sensory neurons exclusively. Many of these lines also show expression within the central nervous system and/or within the epidermis. This is summarised in Table 3.1.

Table 3.1. Summary of expression patterns

Expression	No. of lines
CNS and PNS	10
PNS and epidermal	3
CNS and PNS and epidermal	1
sensory neuron only	7

Each line was examined throughout embryonic development to ascertain at what stage expression began in the nervous system. The exact identity of the sensory neurons revealed by each P[GAL4] line was determined based on their position and morphology. The differences in neuronal morphology between certain classes of sensory neuron can be quite subtle however and, when very few sensory neurons are labelled, the relative position of a

single cell can be difficult to determine. As an additional aid in identification, the position of each sensory neuron in relation to the underlying pattern of embryonic muscles was determined using the antibody Mab22c10, which reveals all embryonic sensory neurons (Fujita et al., 1982). This is shown in Figure 3.2.

An overview of the expression pattern observed in each line that labelled sensory neurons is given in Table 3.2.

Table 3.2 Overview of expression pattern of each line showing PNS staining.

DS Line	Embryonic expression pattern	Class of sensory neuron present
DS04	CNS and PNS	ch
DS20	PNS only	ch
DS24	PNS only	ch and es
DS27	CNS and PNS	ch and md
DS35	CNS and PNS	ch and md
DS43	Epidermal and PNS	*
DS44	CNS and PNS	ch
DS46	CNS and PNS	md
DS47	CNS and PNS	ch, md and es
DS48	Epidermal and PNS	*
DS54	PNS only	ch and md
DS58	Epidermal and PNS	*
DS59	CNS and PNS	ch
DS60	PNS only	ch and md
DS69	CNS and PNS and epidermal	*
DS72	CNS and PNS	ch and md
DS73	CNS and PNS	ch
DS74	CNS and PNS	ch
DS77	PNS only	ch
DS78	PNS only	ch and md
DS97	PNS only	ch and es

* - indicates sensory neuron staining pattern unresolved due to large regions of epidermal staining

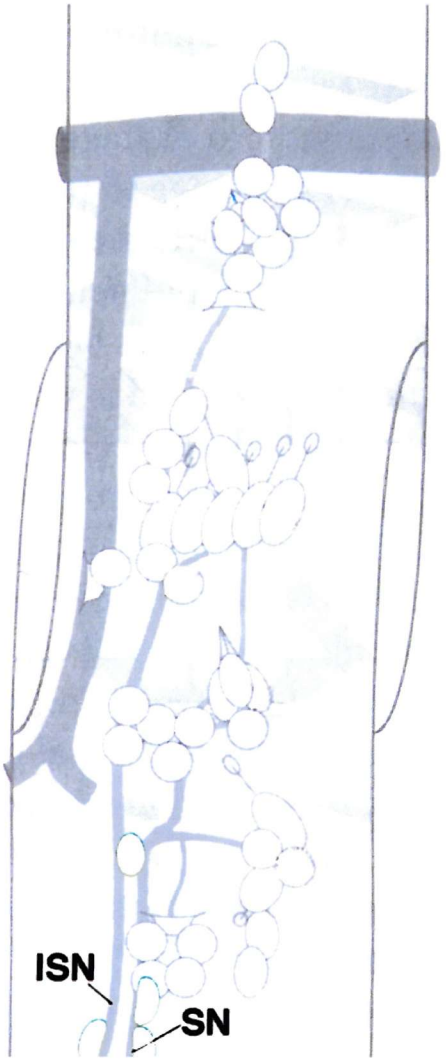


Figure 3.2 The embryonic sensory nervous system in relation to the embryonic musculature. The positions of the sensory neurons in relation to the pattern of embryonic muscles and the trachea are shown in schematic form. Adapted from (Jones, 2001). Anterior is to the left and dorsal is up in this and all subsequent figures, unless otherwise stated. ISN - intersegmental nerve, SN - segmental nerve.

The expression patterns of these lines can be broadly sub-divided into different classes depending on the types of neuron present, as summarised in Table 3.3. Only those lines where the different classes of neurons present can be unambiguously identified have been included in this table. Lines where sensory neurons are present but large areas of the periphery are obscured by epidermal staining have therefore been excluded. In these cases it is unclear whether additional sensory neurons are hidden by the epidermal expression.

Table 3.3 Class of sensory neuron showing expression

Expression	No. of lines
ch, md and es	1
ch and md	6
ch and es	2
md and es	0
ch	7
md	1
es	0

A large number of these lines label chordotonal neurons. Of the 19 lines where the pattern of sensory nervous system expression was clearly visible, 18 show expression in chordotonal neurons. Eight of these 19 lines show a restricted pattern of sensory nervous system expression that is limited to one class/type of sensory neuron. Of these 8, 7 exclusively label chordotonal neurons. Conversely, very few lines show expression in external sensory neurons.

Each of the 21 lines that showed expression in embryonic sensory neurons will now be described in detail. These descriptions carry a caveat however. For each line the predominant staining pattern will be described but, as has been noted previously for P[GAL4] enhancer-trap lines (Lin et al., 1995), there can be some degree of variability in the number of neurons stained between segments. Occasionally a stray cell was present in one segment of one individual that was not observed in any other embryos of that line. This degree of heterogeneity in the expression pattern of P[GAL4] enhancer-trap lines is an important consideration. If such lines are to be used as misexpression vectors, it will always be necessary to verify the exact complement of cells in which the GAL4 protein is active. This can be achieved through co-expression of a reporter gene such as GFP.

3.4.2 Description of individual P[GAL4] enhancer-trap expression patterns

DS04

Pattern of expression

DS04 labels a subset of chordotonal neurons in the embryonic sensory nervous system. The line reveals v'ch1, vchA and vchB but only one of the five lch5 chordotonal neurons, lch5.1, is present (Figure 3.3 C, D). Expression in these cells is very strong and the dendrites of the chordotonal neurons can be clearly seen projecting into the scolopales (Figure 3.3 C, D). There is also a large number of cells expressing within the central nervous system. A stereotyped, segmentally repeated array of neuroblasts and central neurons are labelled (Figure 3.3 A), including axons projecting along the commissures of each side of the ventral nerve cord (data not shown).

Timing of expression

Expression does not begin in the sensory nervous system until stage 16.

DS20

Pattern of expression

The expression of DS20 is restricted to just two neurons per hemisegment in the embryonic peripheral nervous system. Only two of the lch5 chordotonal neurons are labelled (Figure 3.4 B, C). Exactly which chordotonal neurons are stained can vary. Often it is lch5.2 and lch5.4 that are labelled, although different pairs of chordotonal neurons do also appear, as shown in Figure 3.4 B where, on the left, lch5.1 and lch5.5 neuron are present while on the right it is lch5.2 and lch5.4 that are revealed. There is also expression in a paired posterior sense organ (Figure 3.4 E, arrow) and in a stray cell, probably of epidermal origin, just dorsal and posterior to lch5 (Figure 3.4 C, D, arrow).

Timing of expression

Expression begins in these cells at stage 13, after germ band shortening has been completed but prior to dorsal closure.

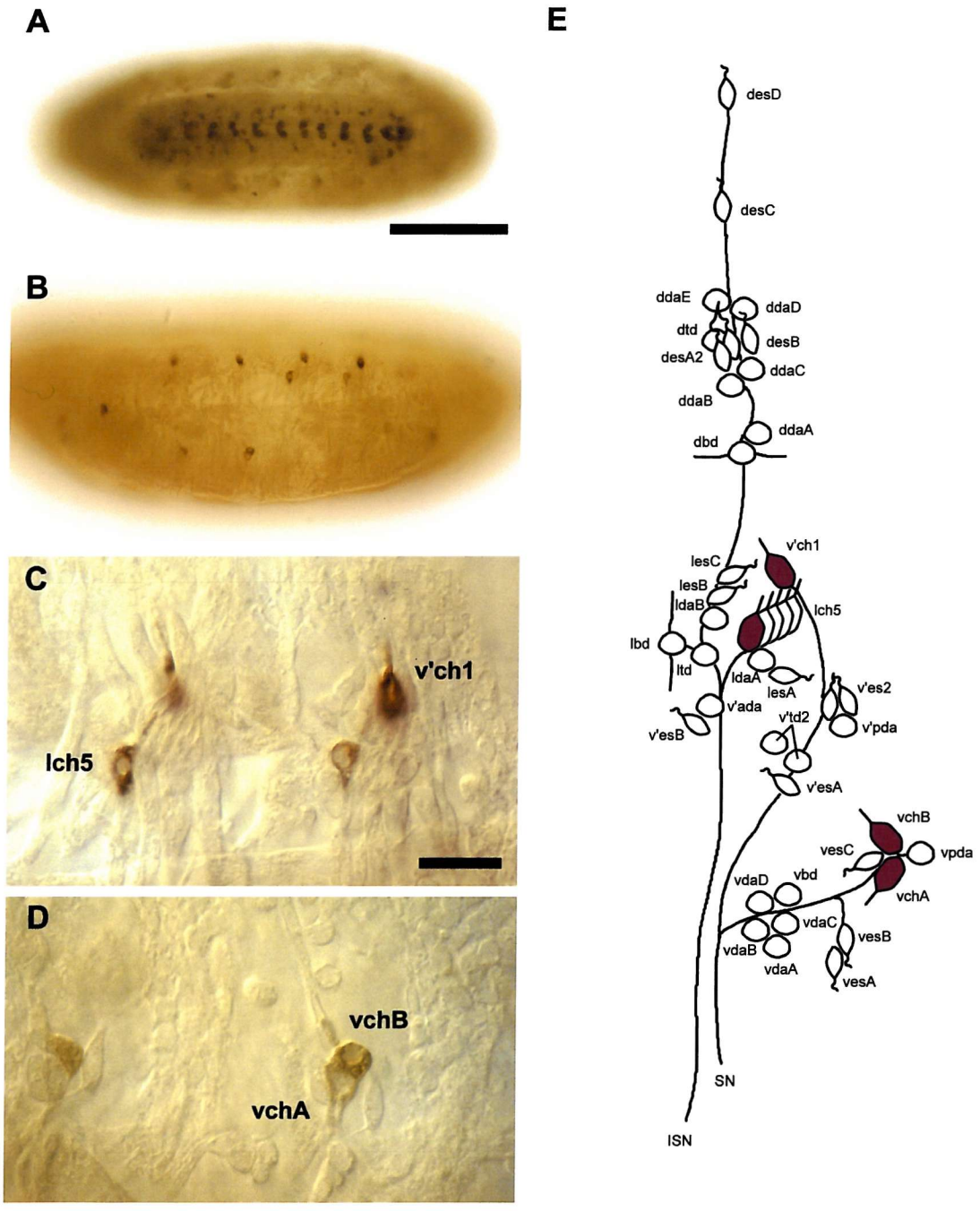


Figure 3.3 DS04 Expression Pattern. A. Ventral view showing expression within the ventral nerve cord. **B.** Lateral view showing expression in the periphery. **C.** In the lateral region a single lch5 neuron, lch5.1, and v'ch1 are labelled. **D.** In the ventral region vchA and vchB are frequently labelled. **E.** Schematic representation of the expression pattern of DS04. (A-B) Scale bar 0.1mm. (C-D) Scale Bar 0.01mm

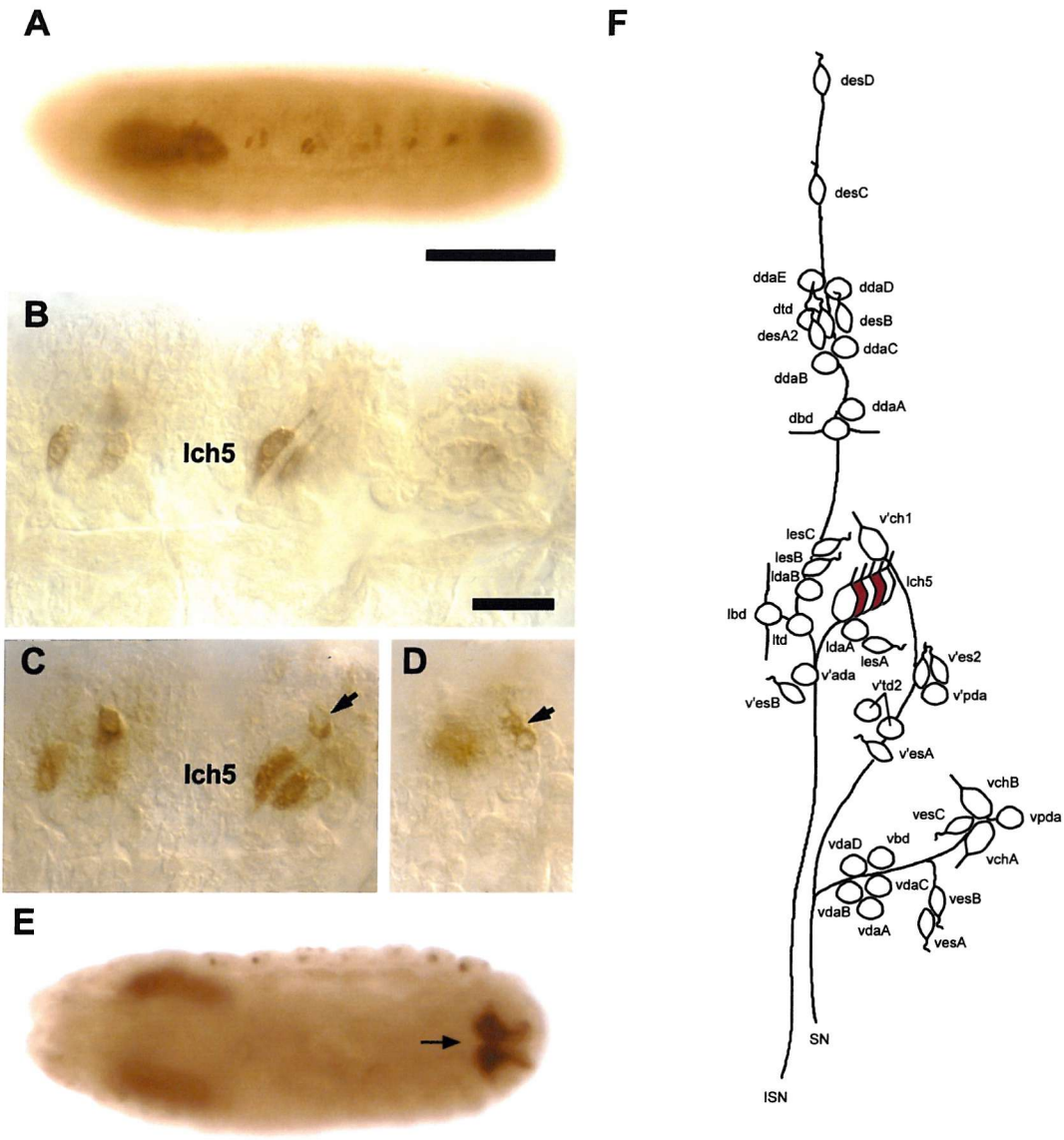


Figure 3.4 DS20 Expression Pattern. A. Lateral view showing peripheral expression. Strong expression is also visible in the salivary glands. **B.** Two lch5 neurons are labelled in each abdominal hemisegment. In the anterior-most segment shown lch5.1 and lch5.4 are labelled while in the next segment lch5.1 and lch5.3 are labelled. **C.** A single cell that is slightly dorsal to the lch5 is also stained (arrow) and is shown again in **D. E.** Dorsal view showing expression in a paired posterior sense organ and the salivary glands. **F.** Schematic representation of the expression pattern of DS20. (A, E) Scale bar 0.1mm. (B-D) Scale bar 0.01mm.

DS24

Pattern of expression

Two or three of the five lch5 chordotonal neurons are strongly stained in each abdominal segment. These can be identified as lch5.1, lch5.4, and generally lch5.3 is also included (Figure 3.5 B, C). The ventral chordotonal neurons vchA and vchB are also occasionally labelled (Figure 3.5 B, D). In addition to chordotonal neurons the line also reveals a single cell, situated ventrally and slightly posterior to lch5 (Figure 3.5 B, arrow). This cell is in a position consistent with either v'es2 or v'pda but from its morphology and the angle of the cell body, I would tentatively assign this cell as one of the v'es neurons. In some segments there is also expression in one lateral cell, slightly dorsal to lch5, which from its position would appear to be lesC (Figure 3.5 C, arrow). More dorsally there is a further single neuron, which is one of the dorsal cluster es neurons and could be either desA2 or desB (Figure 3.5 E, arrow). Again, from its position on the slightly more posterior side of the dorsal cluster I would suggest that this neuron is desB, rather than desA2. There is also a small group of dorsal non-neuronal cells staining in each segment (Figure 3.5 E, arrowhead). These cells are much less superficial and are in close association with the tracheae.

Timing of expression

The embryonic pattern of expression begins at stage 15, just after dorsal closure (Figure 3.5 A).

DS27

Pattern of expression

In DS27 expression is seen in both chordotonal and multidendritic sensory neurons but is confined to the ventral and lateral regions of the embryo (Figure 3.6 A). All chordotonal neurons, with the exception of v'ch1, are clearly labelled (Figure 3.6 C, D). A number of multidendritic neurons are also stained, although this does not include any dorsal cluster mds, as no cells more dorsal than lch5 are labelled. The subset of multidendritic neurons in the ventro-lateral region that do express GFP are lbd, both v'td2 neurons, and v'ada; each of which can be identified based on their position within the segment (Figure 3.6 C, D). In many cases

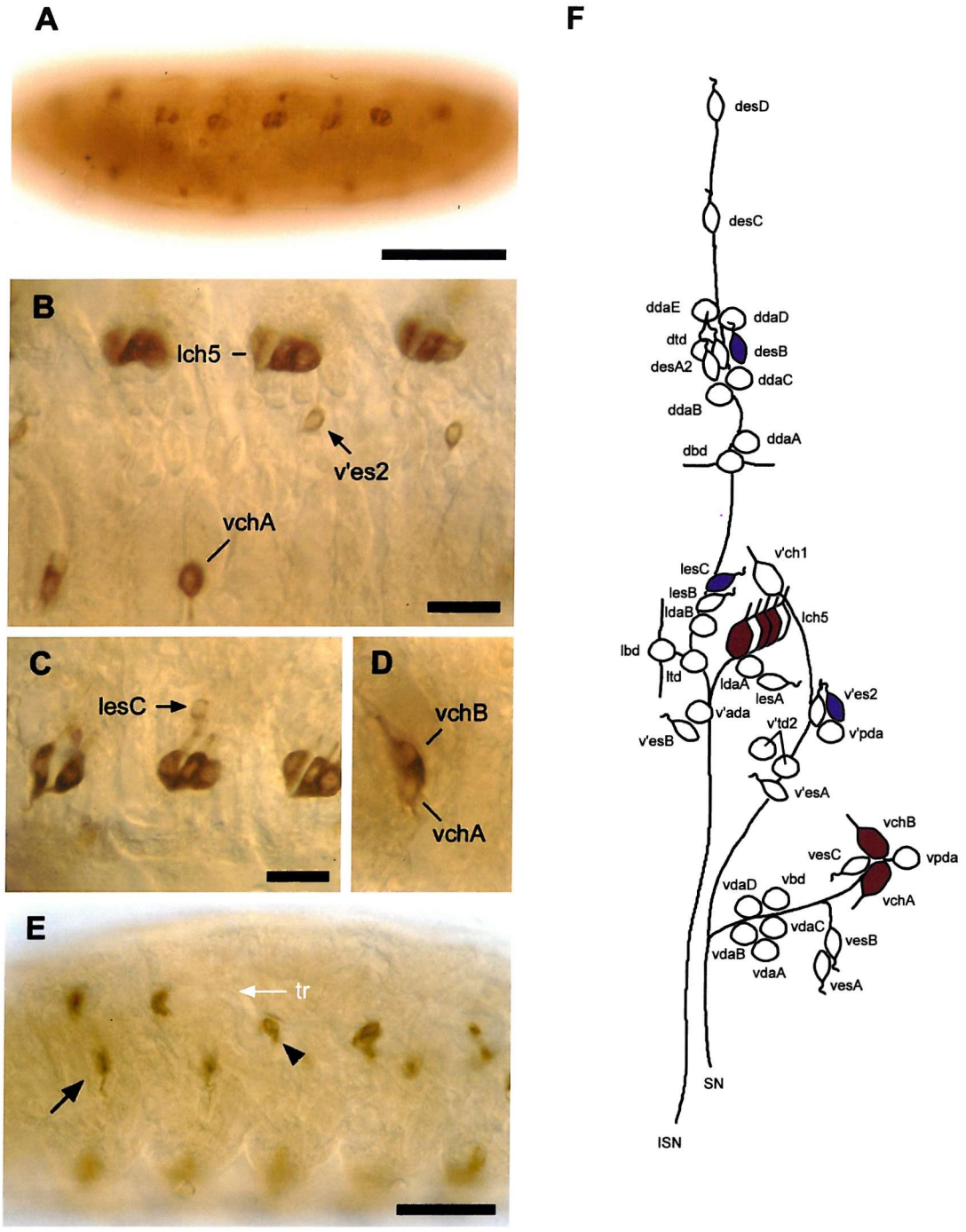


Figure 3.5 DS24 Expression Pattern A. Lateral view showing peripheral expression. **B.** Several lch5 neurons, vchA and v'es2 are labelled. **C.** The lesC neuron, which is slightly dorsal to lch5, frequently shows expression. **D.** In the ventral region vchA and vchB are frequently labelled. **E.** In the dorsal region, a dorsal es neuron (arrow) and a number of cells in close association with the tracheae are stained (arrowhead). The white arrow indicates the trachea (tr). **F.** Schematic representation of the expression pattern of DS24. (A) Scale bar 0.1mm. (B) Scale bar 0.01mm. (C-D) Scale bar 0.01mm. (E) Scale bar 0.03mm.

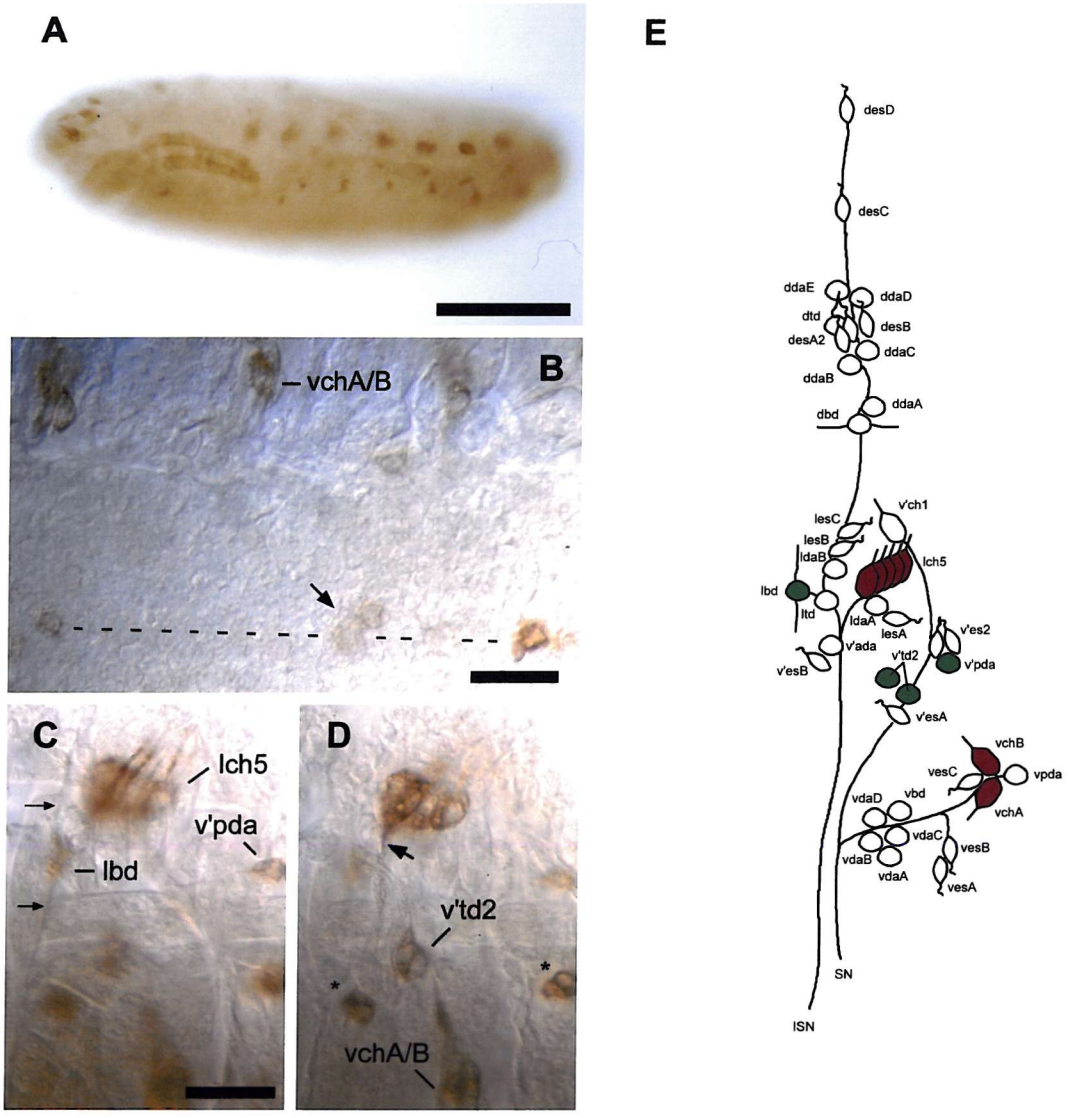


Figure 3.6 DS27 Expression Pattern. A. Lateral view showing peripheral expression **B.** Ventral view showing expression in a discrete number of cells in the ventral nerve cord (arrow) that are situated at the CNS midline (indicated by broken black line). Staining is also apparent in vchA and vchB. **C.** In the lateral region all five lch5 neurons, lbd and v'pda are labelled. Arrows indicate possible staining of the lbd processes along muscle 8. **D.** More ventrally, v'td2 and vchA/B show expression. A cluster of unidentified cells is also labelled in each segment (asterisk). The arrow indicates staining of the lch5 axons. **E.** Schematic representation of the expression pattern of DS24. (A) Scale bar 0.1mm. (B) Scale bar 0.015mm. (C-D) Scale bar 0.01mm.

axons and dendrites are also visualised. The axons of the lch5 neurons can be seen extending down the intersegmental nerve (Figure 3.6 D, arrow). In the case of lbd, the axon and dendrite do appear to be faintly visible along the edge of muscle 8, although this may just be a staining artefact (Figure 3.6 C, arrowheads). A segmentally repeated array of cells in the ventral nerve cord is also labelled (Figure 3.6 B). These cells occupy positions along the midline of the CNS, and could therefore be midline glia.

Timing of expression

Expression begins at stage 16, once the nervous system is fully formed.

DS35

Pattern of expression

In this line the number of cells stained in each segment is extremely variable and much of this expression is very weak. The core staining pattern includes all five lch5 neurons (Figure 3.7 A, B, C), visible in all segments, with a number of additional cells also often being present. The vchA and vchB neurons are occasionally included in the observed expression pattern (Figure 3.7 D, E), but v'ch1 is seldom labelled. Dbd frequently shows expression (Figure 3.7 B), and there is faint staining of all five ventral cluster neurons, including vbd, in some segments (Figure 3.7 E). On rare occassions a single dorsal cluster neuron also shows expression (Figure 3.7 B, asterisk). A further 2-3 v' cells are faintly labelled (Figure 3.7 D, arrow), which I have tentatively identified as v'td2. The third cell that is occasionally observed could be v'esA but the staining is far too faint to make out convincing es neuron morphology. However, expression also accumulates in a mass of small rounded cells at the base of lch5 (Figure 3.7 C, arrowhead). These cells would appear to be the soma sheath cells that enclose the sensory neuron cell body (Hartenstein, 1988), giving rise to the possibility that the additional cells sometimes observed in both the ventral and v' regions of the embryo may have a similar origin and function. As these cells are infrequently present however, I would not consider these cells to be part of the predominant sensory neuron staining pattern of DS35. Expression is not restricted to the periphery as cells within the vnc are also faintly labelled (data not shown). Overall expression in this line is comparatively weak, perhaps partly due to the late onset of expression, and labels a highly variable subset of neurons.

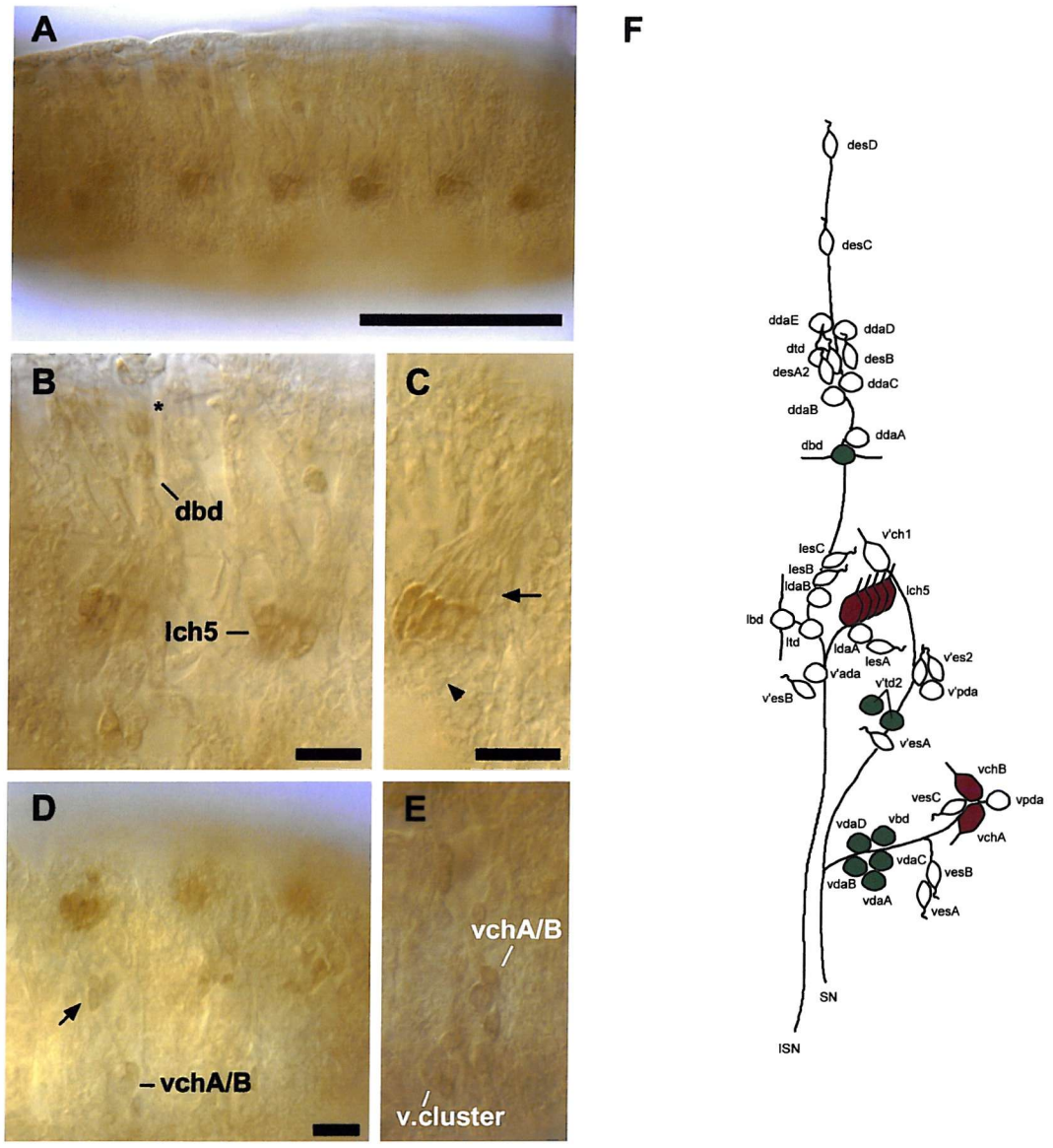


Figure 3.7 DS35 Expression Pattern. A. Lateral view of peripheral expression pattern. **B.** All five lch5 neurons and dbd are labelled, with a dorsal cluster cell being rarely labelled (*). **C.** A closer view of expression in lch5, highlighting the additional cells that are sometimes stained at the base of lch5 (arrowhead). The scolopales are visible (arrow). **D.** Faint expression is visible in a small cluster of v' cells (arrow) and vchA and B. **E.** Weak staining is detected in the ventral cluster neurons and vchA and B. **F.** Schematic representation of the expression pattern of DS35. (A) Scale bar 0.1mm. (B, E) Scale bar 0.01mm. (C) Scale bar 0.01mm. (D) Scale bar 0.01mm.

Timing of expression

Expression begins at stage 15, after dorsal closure is completed.

DS43

Pattern of expression

When initially examined using fluorescence it was clear there was a significant amount of peripheral GFP expression. The line labelled axons and axonal pathways very strongly in a manner reminiscent of Mab22c10 staining and gave rise to the possibility that the line could potentially mark all sensory neurons. Upon closer examination, when GFP reporter gene activity was visualised immunohistochemically, a significant amount of epidermal expression was also present. This staining largely obscured the peripheral nervous system, although the lateral chordotonal organs were still visible. At least one of the lch5 neurons is labelled (Figure 3.8 C, arrows) and an axon from one of the dorsal cluster neurons may also be visible (Figure 3.8 C, arrowhead). Otherwise the extent of epidermal cell staining present precludes identification of any additional sensory neurons present. The pattern of epidermal cells present is non-random and segmentally repeated (Figure 3.8 D). These cells occur in positions within each segment that largely mirror the location of the sensory nervous system. There is also an unusual banded pattern of epidermal cell staining overlying the ventral nerve cord (Figure 3.8 B).

Timing of expression

Expression first becomes visible in the periphery at about stage 13 but also begins in the epidermis at this time, making it difficult to determine the onset of expression in the sensory nervous system (Figure 3.8 A).

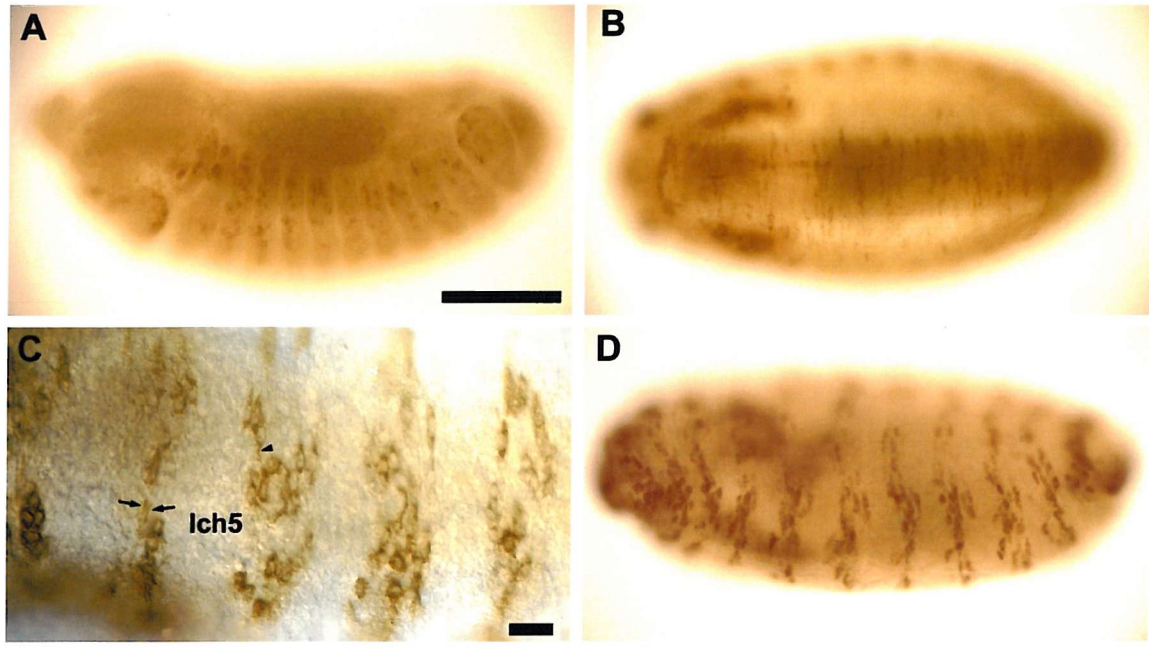


Figure 3.8 DS43 Expression Pattern. A. Lateral view of a stage 13 embryo showing expression beginning in the periphery prior to dorsal closure. **B.** Ventral view showing expression in a banded pattern of epidermal cells overlying the ventral nerve cord. **C.** In the lateral region the dendrites of at least one of the lch5 chordotonal neurons is visible through the epidermal staining (arrows). An axon-like projection is also stained in the vicinity of the dorsal cluster (arrowhead). **D.** Lateral view of a stage 15 embryo, showing epidermal staining. (A-B, D) Scale bar 0.1mm. (C) Scale bar 0.01mm.

DS44

Pattern of expression

DS44 reveals a discrete cluster of peripheral cells and expression is intitially very faint (Figure 3.9 A). The staining surrounds the position of the lch5 neurons, but is diffuse in nature and is not localised to the lch5 (Figure 3.9 E). This expression subsequently becomes strong within a patch of cells that includes all five lch5 chordotonal neurons and the cells that overlie the lateral chordotonal organ (Figure 3.9 C, F). No other sensory neurons seem to be labelled. While the patchy nature of the staining does not preclude the possibility of other cells being stained in the vicinity of lch5, only the outlines and axons of the lch5 neurons were visible when screened fluorescently, indicating that this line is indeed specific for the lch5 chordotonal neurons. There is also a high level of expression throughout the ventral nerve cord and fainter background staining throughout much of the embryo (Figure 3.9 C, D).

Timing of expression

Staining first becomes detectable at early stage 16, where faint expression in a discrete cluster of peripheral cells is visible (Figure 3.9 A). Expression in the ventral nerve cord also begins at this time (Figure 3.9 B). Stronger expression in the periphery does not become apparent until stage 17.

DS46

Pattern of expression

DS46 labels a subset of multidendritic neurons within the embryonic sensory nervous system (Figure 3.10 A, B). In the lateral region of the embryo ldaA and v'pda are both revealed (Figure 3.10 F, G). More dorsally, dbd is labelled (Figure 3.10 D, E, G) and a varying number of dorsal mds, which can include all md neurons in the cluster, show expression (Figure 3.10 D, E). In all embryos examined however, no ventral mds have ever been detected. The line results in fairly high levels of expression, as the axons of these neurons are often clearly visible (Figure 3.10 D, E). DS46 also labels a small number of cells within the vnc (data not shown) and a line of cells at the dorsalmost edge of the embryo (Figure 3.10 C). These cells cannot be involved in dorsal closure as expression does not begin until after dorsal closure is completed and are also unlikely to be the dorsal heart cells as comparision with the Even-

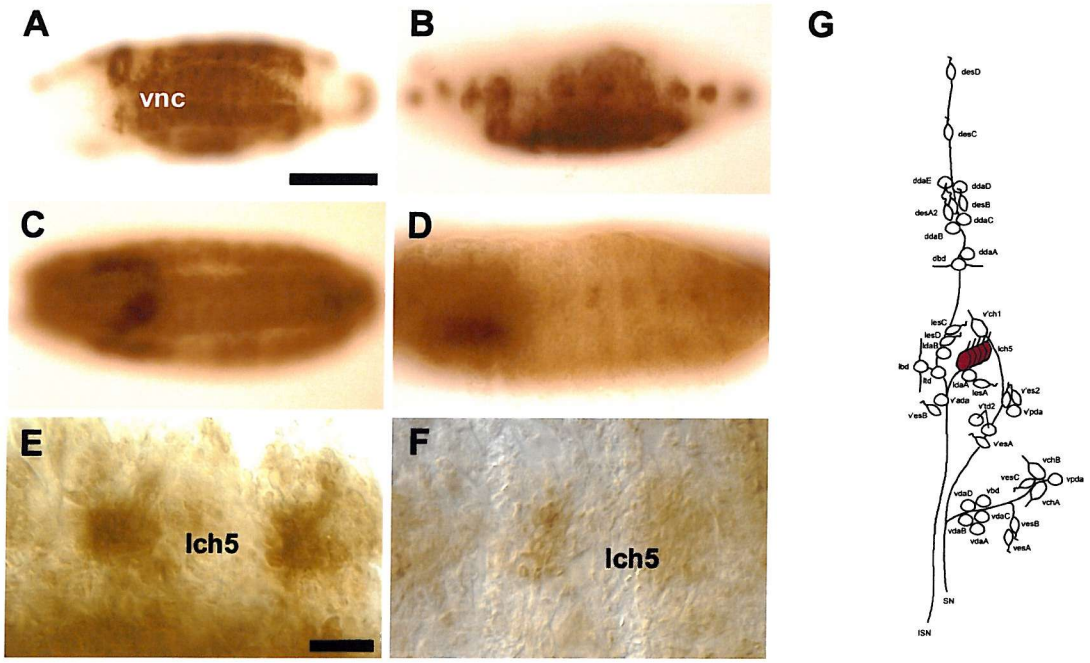


Figure 3.9 DS44 Expression pattern. A. Ventral view of a stage 17 embryo, showing expression in the ventral nerve cord. **B.** Lateral view of a stage 17 embryo showing expression in the periphery. **C.** Ventral view of a stage 16 embryo, showing faint expression within the ventral nerve cord. **D.** Lateral view of a stage 16 embryo showing faint expression in a lateral cluster of cells in each abdominal segment. **E.** Stage 17 embryo showing strong expression surrounding the lch5. **F.** Stage 16 embryo showing faint staining surrounding the lch5. **G.** Schematic representation of the DS44 staining pattern. (A-D) Scale bar 0.1mm. (D-E) Scale bar 0.01mm.

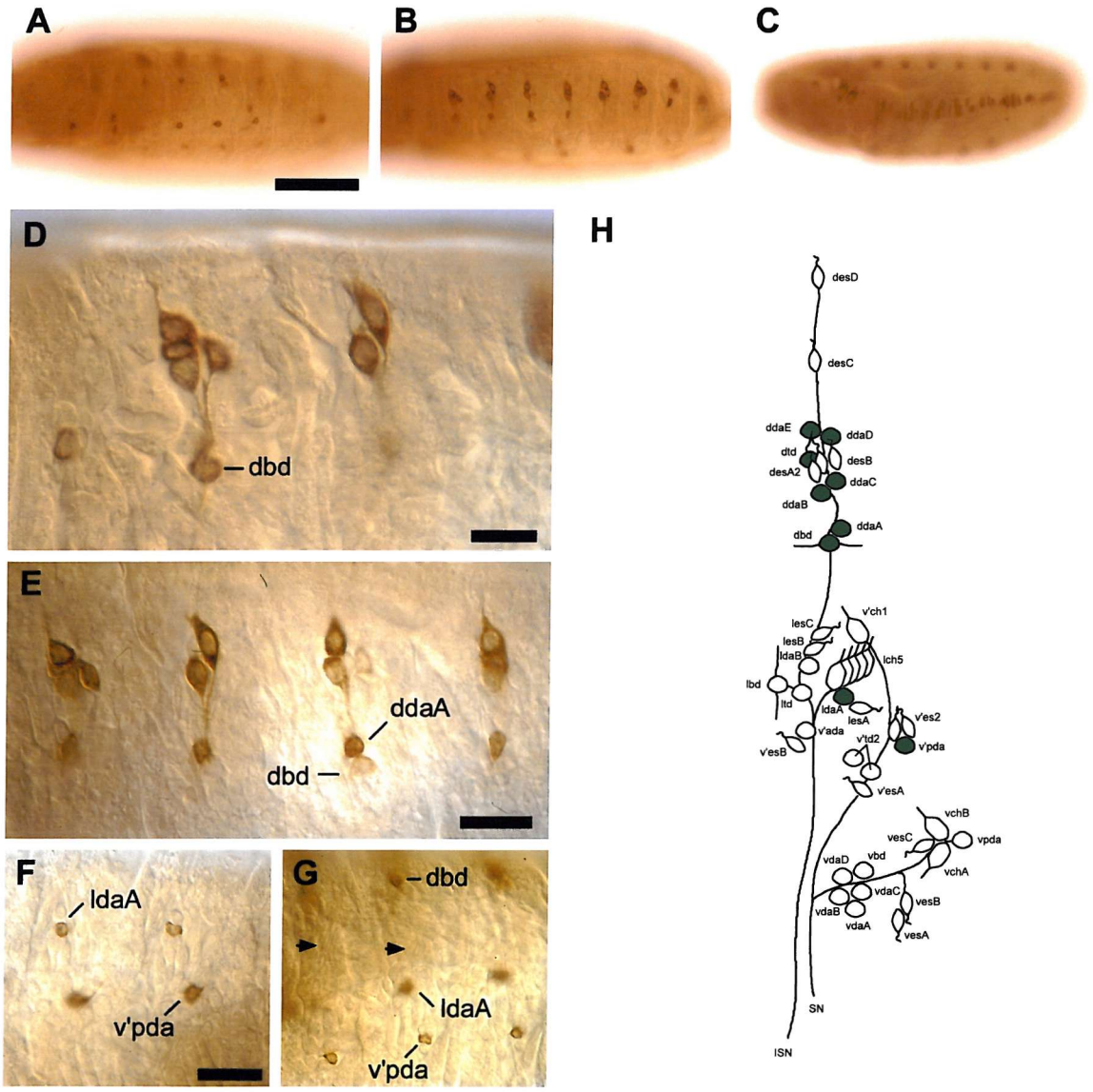


Figure 3.10 DS46 Expression Pattern. A. Ventro-lateral view of peripheral expression pattern. **B.** Dorso-lateral view of peripheral expression pattern. **C.** Dorsal view showing expression in a line of cells at the dorsalmost edge of the embryo. **D. E.** Expression is apparent in dbd and a variable number of dorsal cluster md neurons. **F. G.** In the lateral region, ldaA and v'pda are labelled. The lch5 scolopale cells are visible (arrowheads in **G**). **H.** Schematic representation of the DS46 staining pattern. (A-C) Scale bar 0.1mm. (D) Scale bar 0.007mm. (E) Scale bar 0.007mm (F-G) Scale bar 0.02mm

skipped antibody staining pattern (Figure 2.2C), which does label the paracardial cells, shows that the cells labelled in DS46 are far more superficial and have a different morphology. These cells could be neurosecretory cells however which, interestingly, are also multipolar (McIver, 1981).

Timing of expression

Expression does not begin until stage 15, after dorsal closure. By this stage the embryonic sensory nervous system is fully formed.

DS47

Pattern of expression

DS46 labels all embryonic sensory neurons (Figure 3.11 A, C, D, E). There is also expression within a subset of CNS cells (Figure 3.11 B, arrows) and a number of non-neuronal peripheral cells (Figure 3.11 E, arrowheads).

Timing of expression

Expression begins at stage 15, after dorsal closure.

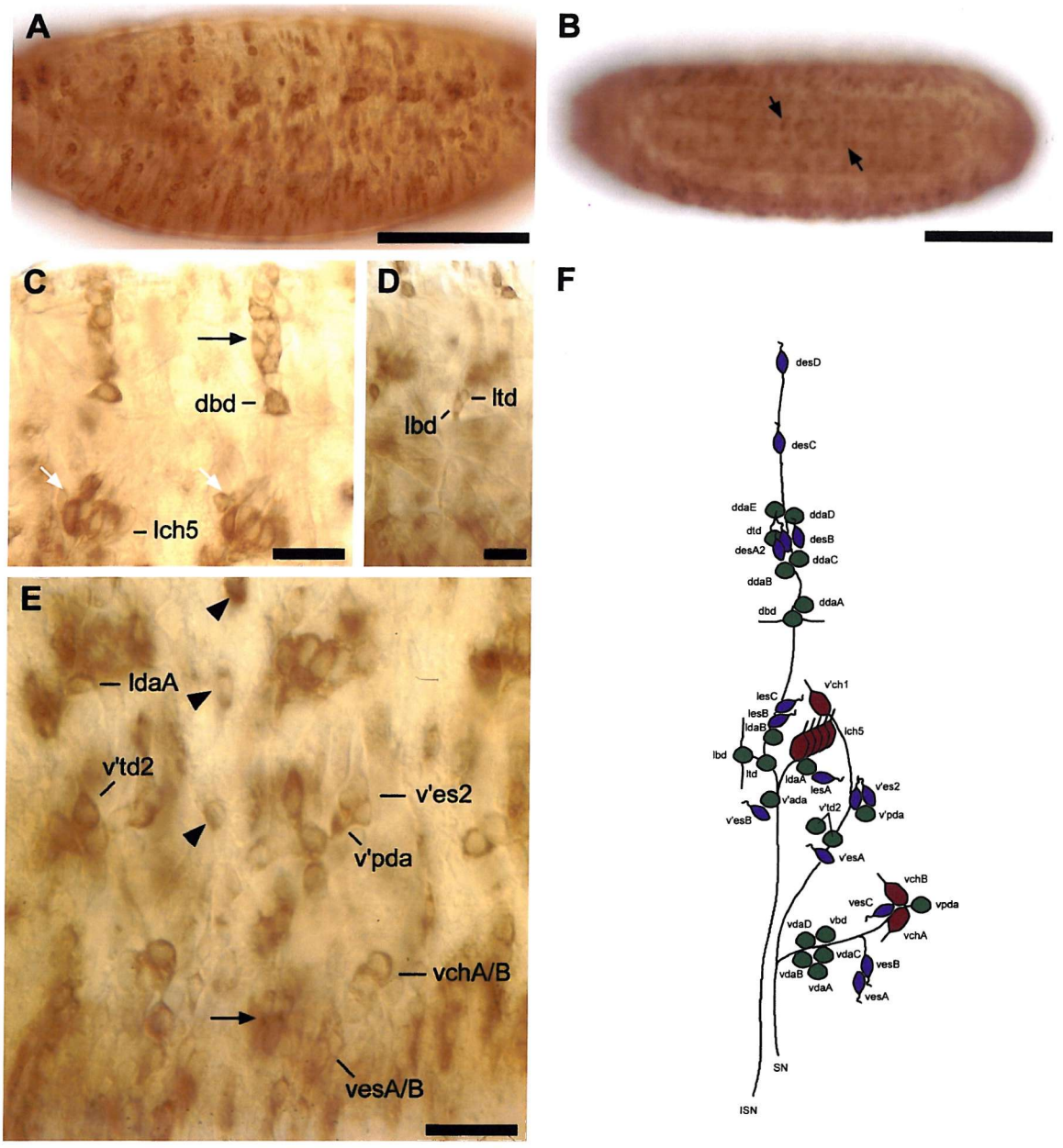


Figure 3.11 DS47 Expression Pattern. A. Lateral view showing peripheral expression. **B.** Ventral view showing expression in a subset of CNS cells (arrows). **C.** All cells in the dorsal region are labelled. Arrow indicates staining in all dorsal cluster neurons. More laterally, all five lch5 neurons and all the neurons that lie adjacent to lch5 (white arrow) show expression. These are ldaB, lesB and lesC. **D.** Lbd and ltd are labelled. **E.** All sensory neurons in the lateral, ventro-lateral, and ventral region show expression. The black arrow indicates the position of the ventral cluster of neurons containing vda A-D and vbd. Arrowheads indicate additional peripheral staining of non-neuronal cells. **F.** Schematic representation of the expression pattern of DS47. (A) Scale bar 0.1mm. (B) Scale bar 0.1mm. (C) Scale bar 0.01mm. (D) Scale bar 0.01mm. (E) Scale bar 0.01mm.

DS54

Pattern of expression

DS54 only shows expression in one lch5 neuron, which often corresponds to lch5.1, and this neuron is clearly labelled in the majority of abdominal segments (Figure 3.12A). In one embryo expression was also detected in dbd, but in one segment only. The extremely low incidence of dbd staining would suggest that dbd is not part of the normal expression pattern of DS54. A number of ventral epidermal cells are occasionally stained in some segments. In addition, one of the cephalic sense organs is labelled (Figure 3.12 A, black arrow)and a number of cells are stained within the CNS (Figure 3.12 A, white arrows)

Timing of expression

This expression begins at stage 16.

DS58

Pattern of expression

Staining in abdominal segments first becomes visible in a small patch of cells in a lateral position in each segment (Figure 3.13 B, arrow). This small cluster of cells foreshadows the large patch of epidermal cell staining visible in each segment at later stages (Figure 3.13 C). This large cluster of cells overlies lch5. Epidermal staining completely obscures lch5 however and it is impossible to determine whether lch5 is incorporated in the expression pattern. Occasionally one or two stray peripheral cells are additionally labelled in some segments but no cells show expression in the CNS. A small group of cells is labelled in the head segments (Figure 3.13 A, arrow), and this structure continues to stain throughout embryonic development.

Timing of expression

Expression appears first in the presumptive head region at stage 12, during germ band extension (Figure 3.13 A). Later, at stage 14, expression begins in the periphery (Figure 3.13 B).

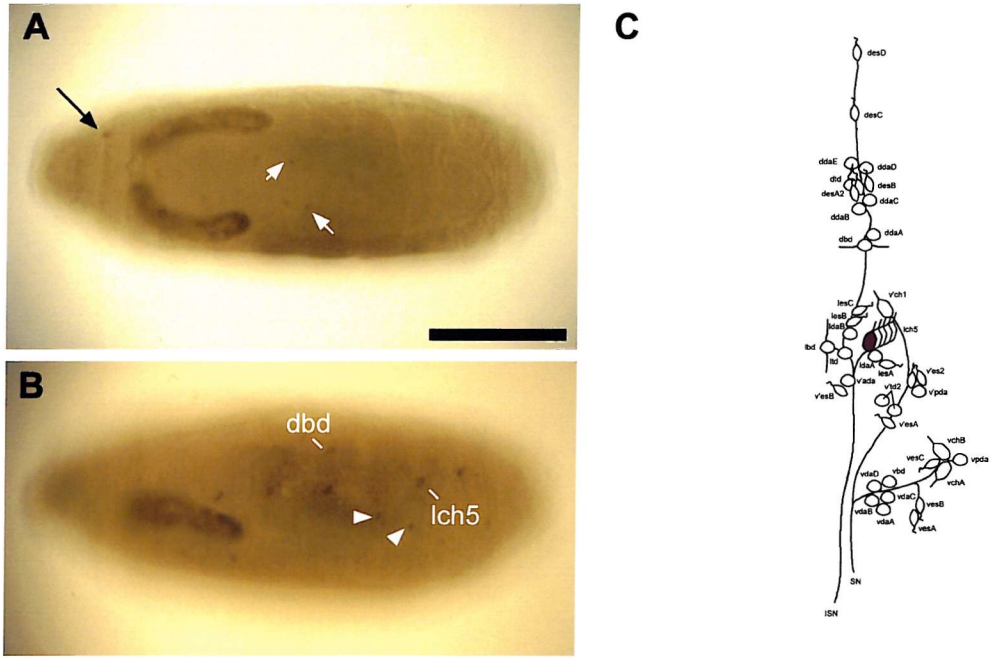


Figure 3.12 DS54 Expression Pattern. A. Ventral view showing expression in a subset of cells within the ventral nerve cord (white arrows) and in a cephalic sense organ (black arrow). **B.** Lateral view showing expression in a single lch5 neuron in each abdominal segment, lch5.1. A number of epidermal cells are labelled in the ventral region (arrowheads) and dbd is labelled in one segment. **C.** Schematic representation of the expression pattern of DS54. (A-B) Scale bar 0.1mm.

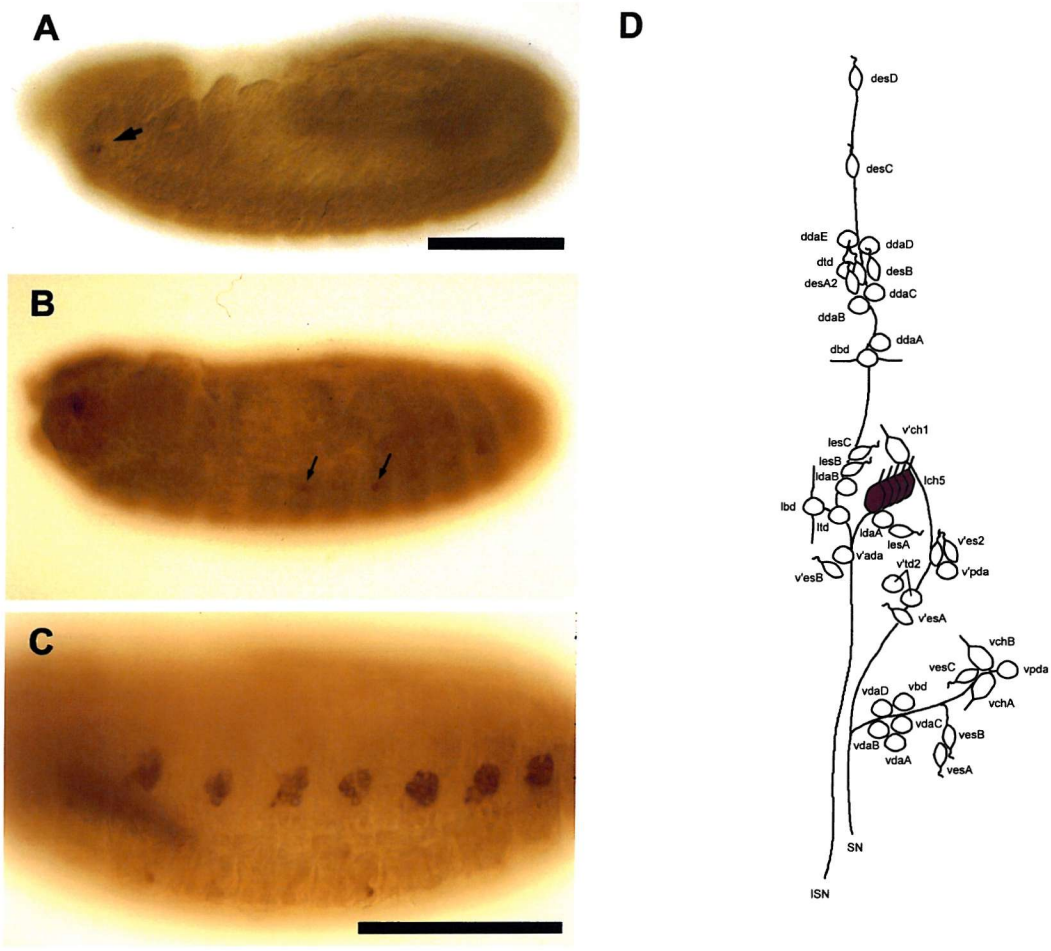


Figure 3.13 DS58 Expression Pattern. A. Lateral view of a stage 12 embryo. A small group of cells in the presumptive head region are labelled. **B.** Lateral view of a stage 14 embryo. A lateral patch of cells show expression. **C.** Stage 16 embryo showing expression in a large cluster of epidermal cells overlying lch5. **D.** Schematic representation of the DS58 staining pattern. (A-B) Scale bar 0.1mm. (C) Scale bar 0.1mm.

DS59

Sensory nervous system expression in DS59 encompasses a small number of chordotonal neurons. A few lch5 neurons are labelled in abdominal segments (Figure 3.14 A, C) but no expression was visible in vchA, B or v'ch1. There seems to be slight variation between segments in the number of lch5 cells visualised and the identity of the labelled neurons. In general between one and three lch5 neurons are observed to be stained. There is also sensory expression in a number of unidentified anterior sense organs (Figure 3.14 B, black arrow). The majority of embryos examined clearly showed expression in the salivary glands and CNS. Within the ventral nerve cord DS59 appears to label a subset of cells almost at the ventral midline (Figure 3.14 B, white arrow). There is also strong background staining within the gut.

Timing of expression

Staining is apparent in the ventral nerve cord from about stage 14 onwards. Expression does not become apparent in the sensory nervous system however until the end of stage 16

DS60

Pattern of expression

Expression in DS60 is again limited to a very discrete number of cells (Figure 3.15 A). Only chordotonal neurons are present in the predominant staining pattern, although a single v' multidendritic neuron, v'ada, is sometimes labelled (Figure 3.15 B). Chordotonal neurons from each of the three groups of chordotonal neurons in the embryo are present (Figure 3.15 B). In the ventral position a single ventral chordotonal neuron is observed, vchB. V'ch1 is present and 3-4 of the lch5 neurons, are also labelled. Within lch5 itself, lch5.1 tends to be strongly stained with weaker expression in the remaining lateral chordotonal neurons. There is no further peripheral expression and there is no detectable staining in the CNS.

Timing of expression

Expression begins at stage 16, near the end of embryonic development.

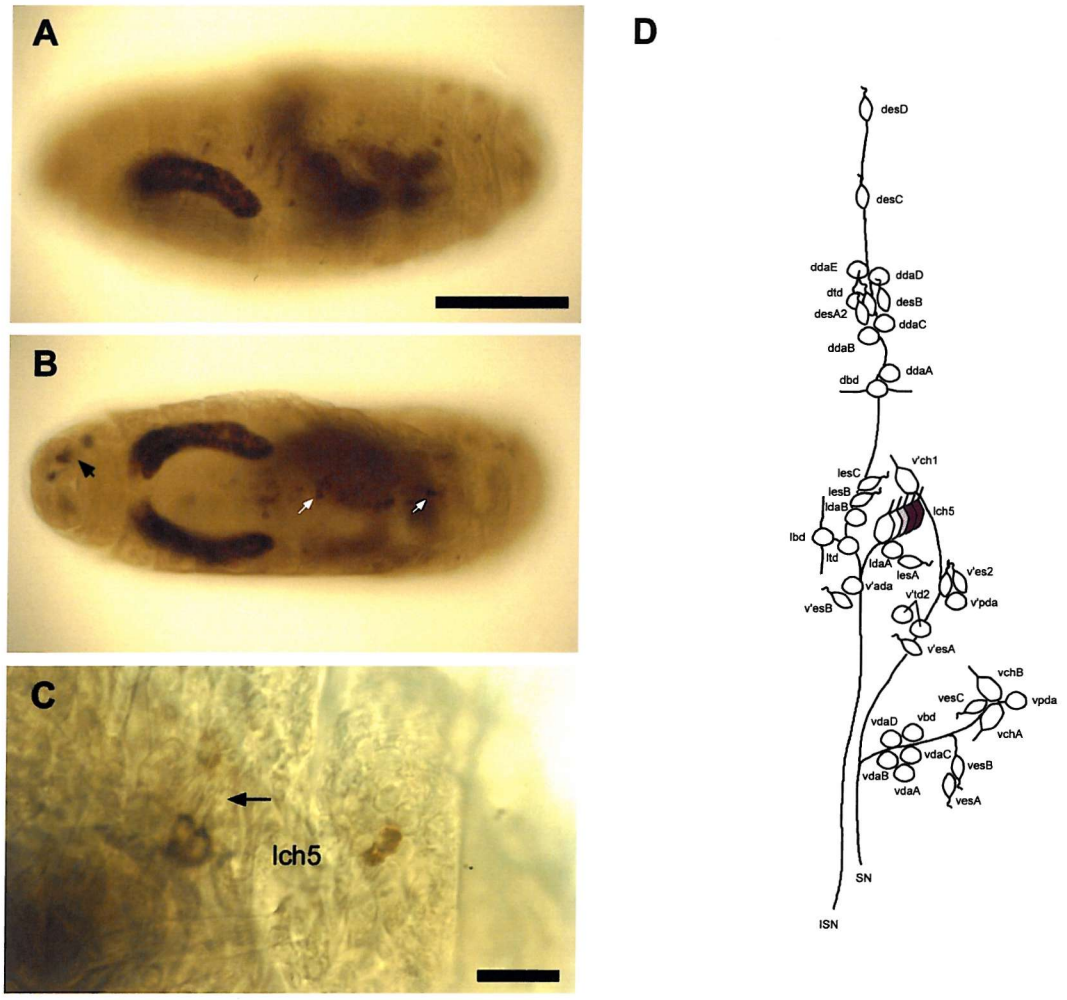


Figure 3.14 DS 59 Expression Pattern. A. Lateral view showing expression in the periphery. Expression is apparent in a small number of lch5 neurons, and in the gut and salivary glands. **B.** Ventral view showing expression in a discrete number of cells in the CNS (white arrows) and in unidentified anterior sense organs (black arrow) **C.** One or two lch5 neurons show expression in each segment. Arrow indicates the position of the scolopale cells, which are visible. **D.** Schematic representation of the DS59 staining pattern. (A-B) Scale bar 0.1mm. (C) Scale bar 0.01mm.

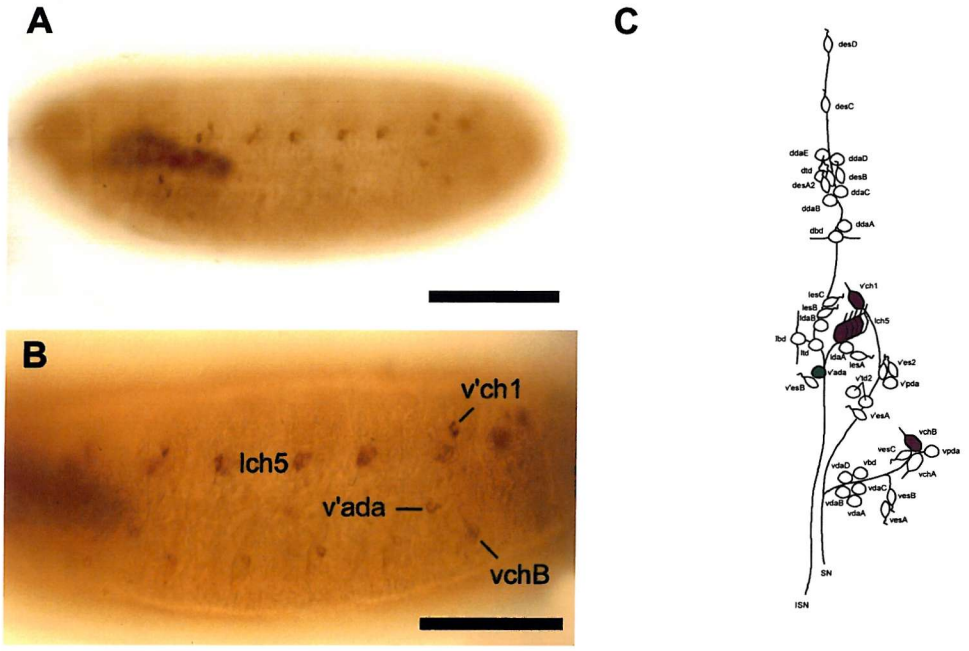


Figure 3.15 DS60 Expression Pattern. A. Lateral view showing peripheral expression. **B.** Expression is apparent in the majority of lch5 neurons, v'ch1, v'ada and vchB. **C.** Schematic representation of the DS60 staining pattern. (A) Scale bar 0.1mm. (B) Scale bar 0.1mm.

DS69

Pattern of expression

There is a significant amount of staining in large clusters of peripheral cells, many of which are epidermal, although some labelled neurons are also evident in these clusters. As in DS43, these epidermal cells form a very distinct, segmentally repeated pattern that overlies a large section of the sensory nervous system. The majority of this staining is dorsal to lch5 and encompasses the dorsal cluster (Figure 3.16 G), with several cells extending as far as the dorsal midline (Figure 3.16 E). Of the neuronal cells showing expression, lch5.1 is clearly visible, as it is not concealed by epidermal staining (Figure 3.16 F). There also appears to be at least one neuron in the dorsal cluster labelled and from its morphology and position, this cell would seem to be an es neuron, possibly one of desA2 (Figure 3.16 F, arrow). The dorsal cluster could contain additional neurons, but these may be occluded by the epidermal cell staining. The extremely dorsal cells labelled are in a position consistent with the dorsalmost es neurons desC and desD, but from the morphology revealed by antibody staining their identity remains ambiguous (Figure 3.16 E, arrow). There are also small clusters of stained cells in the v' region of the embryo that might contain a neuron and a couple of ventral cells expressing in approximately the vchA/B position but the vch neurons themselves are not visible. DS69 also labels a discrete pattern of epidermal cells covering the ventral nerve cord and an unidentified internal structure in the same position (Figure 3.16 C, arrow).

Timing of expression

Peripheral expression, including epidermal expression, becomes evident during the completion of germ band shortening (Figure 3.16 A), at stage 12-13.

DS72

Pattern of expression

There is expression in all five lch5 chordotonal neurons in all segments, although the strength of staining varies somewhat between different lch5 neurons in different segments (Figure 3.17 B, C, D). Staining is also manifest in vchA in the majority of segments (Figure 3.17 D). Several multidendritic neurons are often labelled in a number of segments but expression in these cells is much less penetrant, as they are only ever stained in one or two abdominal

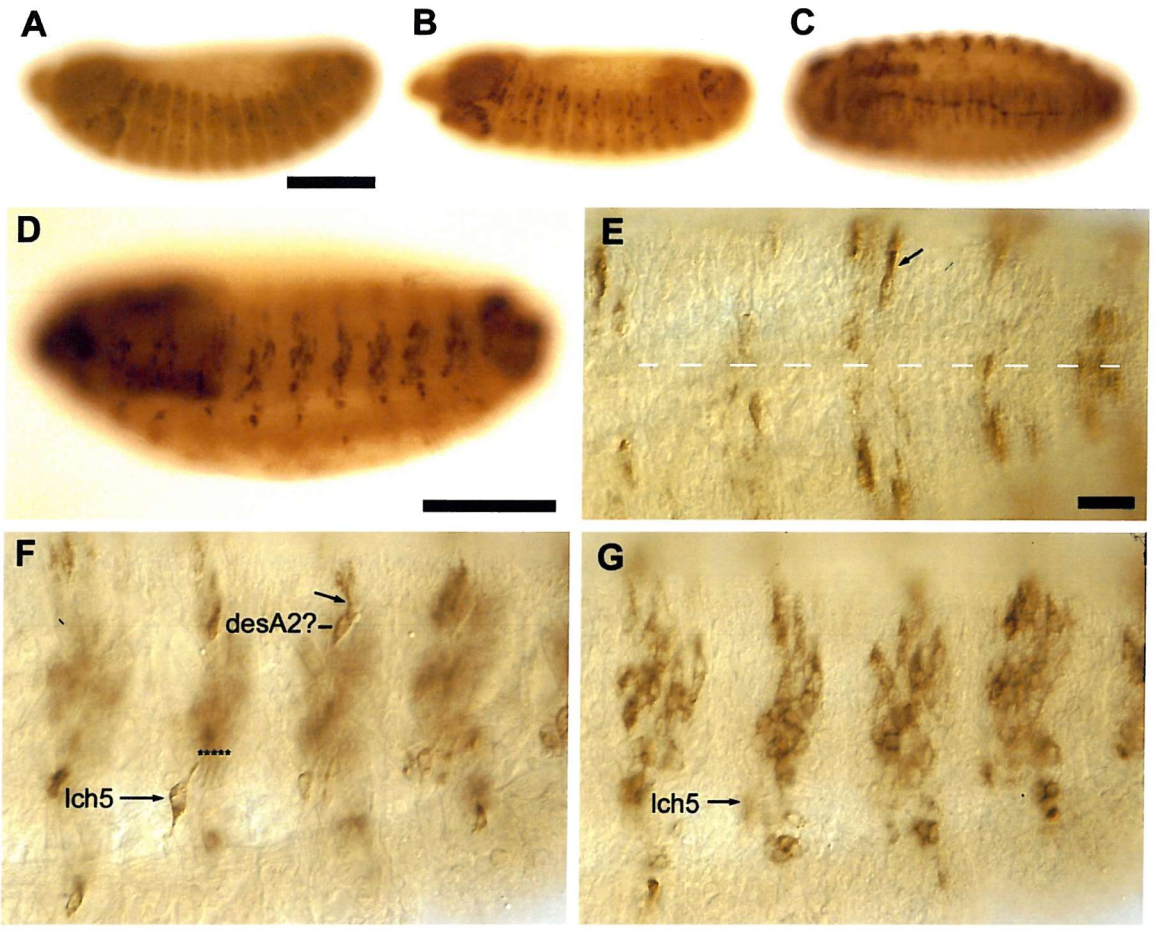


Figure 3.16 DS69 Expression Pattern. A. Lateral view of a stage 13 embryo. **B.** Lateral view of a stage 14 embryo. **C.** Ventral view of a stage 15 embryo showing expression overlying the ventral nerve cord. **D.** Lateral view of peripheral expression pattern in a stage 15 embryo. **E.** A number of cells are stained close to the dorsal midline (indicated by broken white line). Arrow indicates possible desC/desD axon. **F.** A single lch5 neuron is labelled, although all lch5 neurons are visible (asterisks). DesA2 is also possibly labelled (arrow indicates possible dendrite). **G.** A large cluster of epidermal cells are labelled dorsal to lch5. (A-C) Scale bar 0.1mm. (D) Scale bar 0.1mm. (E-G) Scale bar 0.01mm.

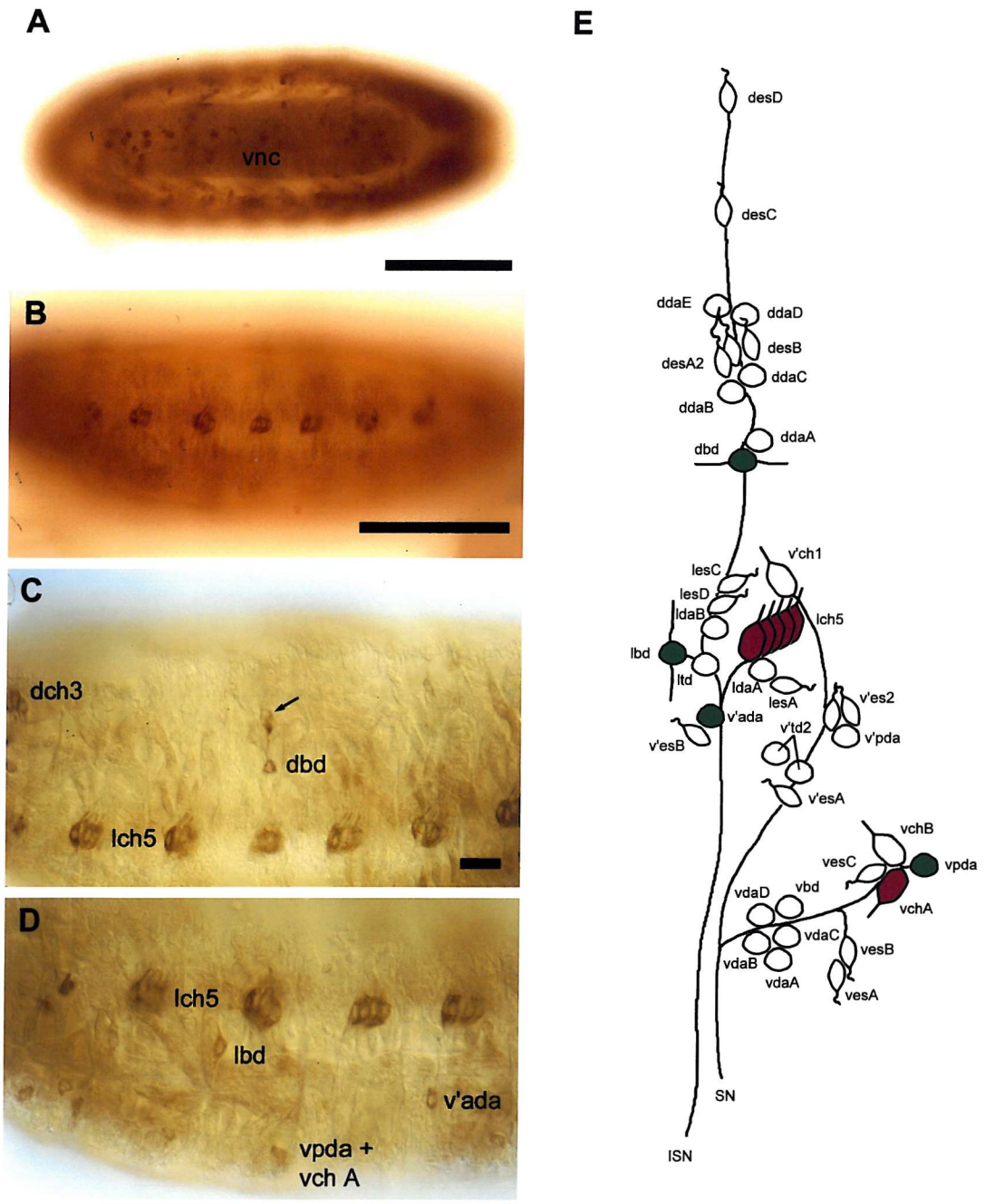


Figure 3.17 DS 72 Expression Pattern. A. Ventral view showing expression in CNS. **B.** Lateral view showing peripheral expression. **C.** Expression is apparent in lch5 and in the dorsal region dbd is often labelled. Two dorsal cluster cells very occasionally show expression (arrow). Staining of dch3 in the thoracic segments is also shown. **D.** In the lateral and ventral regions lch5, lbd, v'ada, vpda and vchA are stained. **E.** Schematic representation of the DS72 staining pattern. (A) Scale bar 0.1mm. (B) Scale bar 0.1mm. (C-D) Scale bar 0.01mm.

embryonic segments. These include two of the three bipolar multidendritic neurons, namely dbd (Figure 3.17 C) and lbd (Figure 3.17 D), and two dendritic arborisation neurons, vpda and v'ada. There is also occasional staining of one to two dorsal cluster cells (Figure 3.17 C, arrows). The compact nature of the dorsal cluster means that cells cannot be unambiguously identified based on position alone. Expression in these cells is very faint, but from the morphology that can be visualised and the general position of these cells I would suggest these neurons could be ddaB and desB. The labelling of these cells is extremely rare however, and has not been included in the general staining pattern of DS72. DS72 also reveals a number of cells within the ventral nerve cord (Figure 3.17 A).

Timing of expression

Expression begins around the same time in both the sensory and central nervous system, at about stage 16.

DS73

Pattern of expression

DS 73 labels a subset of chordotonal neurons. The exact number of chordotonal neurons visualised is variable, with v'ch1 (Figure 3.18 E) and at least one and generally two lch5 neurons always showing expression (Figure 3.18 D). VchB is also frequently labelled (Figure 3.18 F). All neurons are very faintly stained. As this particular line was processed for embryo staining at the same time as DS69, which shows very strong GFP expression, this faint staining probably reflects a genuine difference in the level of reporter gene activity in this line, rather than a failure of the antibody staining procedure. Expression is also detected in a number of cells in the head region and in a discrete number of CNS cells.

Timing of expression

Expression begins at stage 13 in the cephalic sensory neurons but the abdominal chordotonal neurons and CNS cells only begin to be labelled at stage 16.

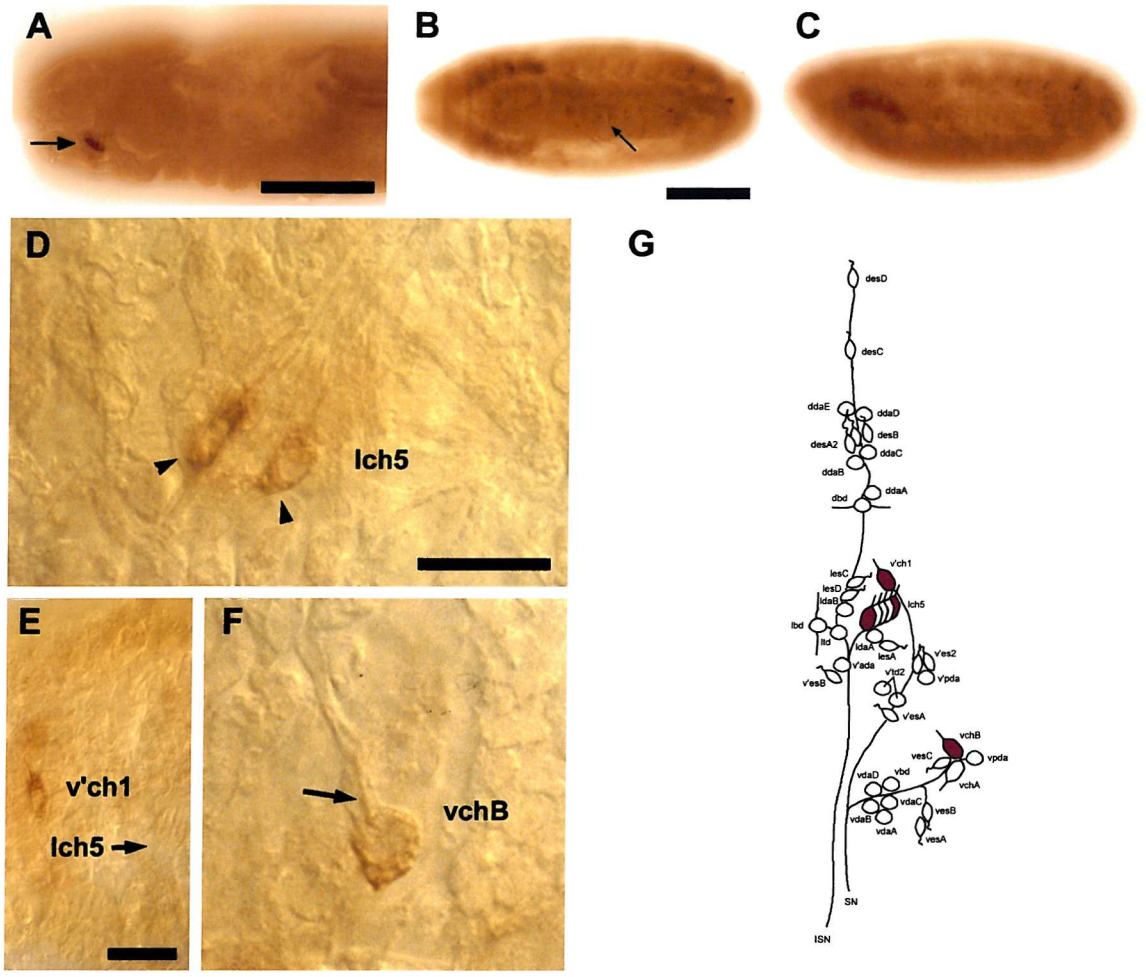


Figure 3.18 DS73 Expression Pattern. A. A number of sensory neurons in the cephalic segments show expression at stage 13 (arrow). **B.** Ventral view showing ventral nerve cord expression (arrow). **C.** Lateral view showing peripheral expression. **D.** Faint expression is visible in lch5.1 and lch5.5, arrowheads. **E.** Expression is apparent in v'ch1. **F.** In the ventral region vchB is labelled. **G.** Schematic representation of the DS73 staining pattern. (A) Scale bar 0.1mm. (B-C) Scale bar 0.1mm. (D, F) Scale bar 0.01mm. (E) Scale bar 0.01mm.

DS74

Pattern of expression

Expression begins in a lateral cluster of cells that overlies lch5 (Figure 3.19 B, C). All five chordotonal neurons are faintly labelled (Figure 3.19 C). There is also stronger expression throughout the CNS (Figure 3.19 A, B) but no other sensory neurons are visible and no other cells appear to be stained. The extremely late onset of expression in the sensory nervous system, which does not appear until just prior to hatching, suggests that this staining should perhaps be considered to represent larval rather than embryonic GAL4 expression in these neurons.

Timing of expression

Expression begins in the CNS at about stage 15, but does not become apparent in the periphery until late stage 16, early stage 17.

DS 77

Pattern of expression

Expression is faintly detected in all five chordotonal neurons of lch5 (Figure 3.20 D, E). A single dorsal cell is also present in some segments (Figure 3.20 D, white arrow). Although it is in the vicinity of the dorsal cluster, which can be visualised using Differential Interference Contrast (DIC), it is clearly not part of the dorsal cluster and lies much further below the surface, and is therefore not a sensory neuron. Also labelled is a paired anterior sense organ in the presumptive head region (Figure 3.20 C, D black arrows). This would appear to be the dorsal organ of the procephalic nervous system (Hartenstein, 1988) and both the cell bodies and axons show expression. Staining is also apparent within the amnioserosa (Figure 3.20 A, B). There is no further expression in the periphery or in the CNS .

Timing of expression

Staining is first detected in amnioserosa cells at about stage 10 (Figure 3.20 A), during germ band extension, and continues until dorsal closure (Figure 3.20 B), at which point the cells are no longer visible. Expression first appears in the procephalic sensory nervous system just

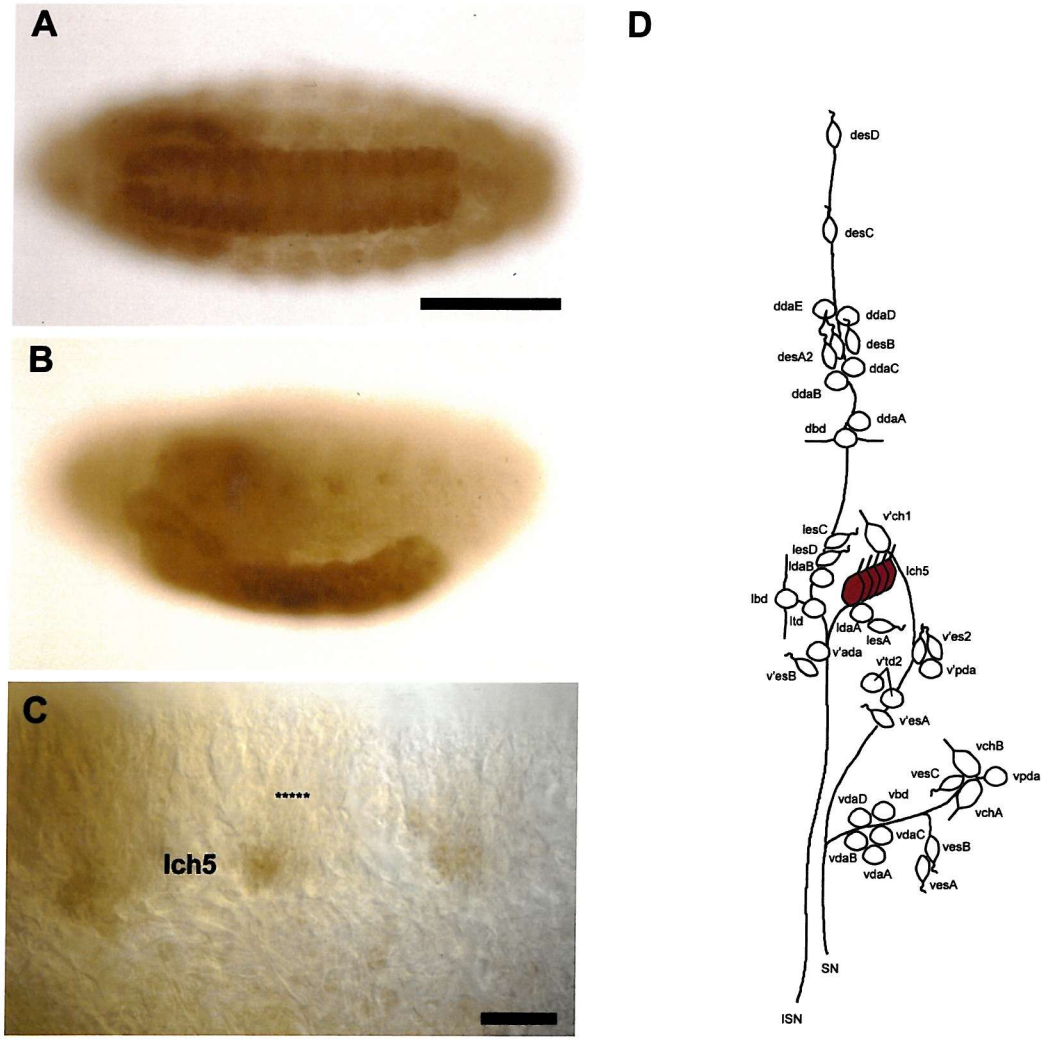


Figure 3.19 DS74 Expression Pattern. A. Ventral view showing ventral nerve cord expression. **B.** Lateral view showing expression in the periphery. **C.** Faint expression is detected in all five lch5 neurons. Asterisks indicate the scolopale cells. **D.** Schematic representation of the DS74 staining pattern. (A-B) Scale bar 0.1mm. (C) Scale bar 0.01mm.

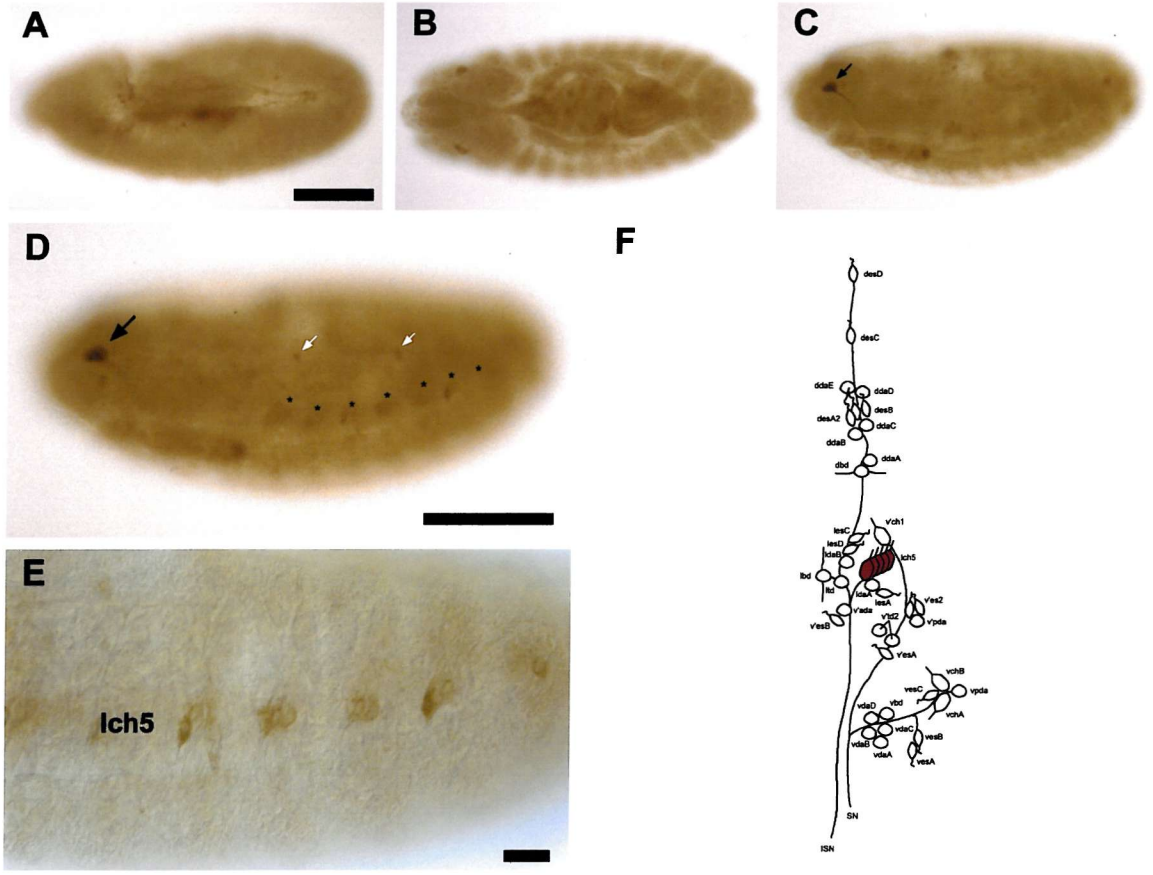


Figure 3.20 DS77 Expression Pattern. A. Stage 10 embryo showing expression in amnioserosa. **B.** Dorsal view of stage 13 embryo. **C.** Lateral view of stage 15 embryo. Expression is visible in the dorsal organ of the procephalic system (arrow, and **D**, black arrow). **D.** Lateral view showing peripheral expression. Asterisks highlights the lch5 staining present in each segment. A white arrow indicates the dorsal cell that is sometimes labelled. **E.** All 5 lch5 neurons are labelled. **F.** Schematic representation of the DS77 staining pattern. (A-C) Scale bar 0.1mm. (D) Scale bar 0.1mm. (E) Scale bar 0.01mm.

prior to dorsal closure and begins to be detected in the chordotonal neurons just after dorsal closure, at about stage 14.

DS78

Pattern of expression

DS78 labels both chordotonal neurons and multidendritic neurons. All eight chordotonal neurons are revealed (Figure 3.21 B, C) and a single multidendritic neuron, v'pda is also often present. The line drives strong expression in these cells and their axons (Figure 3.21 B, arrows) and dendrites (Figure 3.21 C, arrows) are often clearly visible. There is no expression in the central nervous system or in any additional peripheral cells.

Timing of expression

Expression first becomes apparent in the sensory nervous system at stage 16.

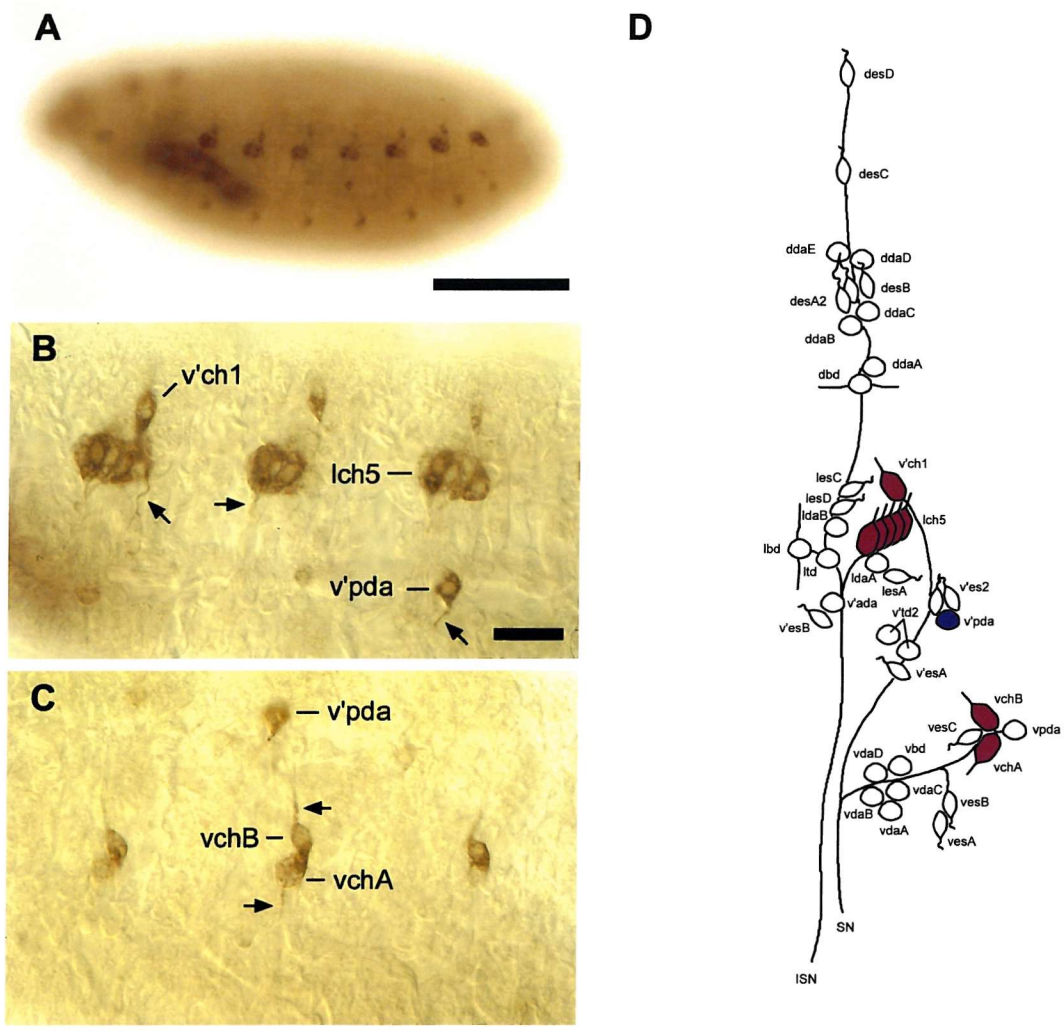


Figure 3.21 DS78 Expression Pattern. A. Lateral view showing peripheral expression. **B.** In the lateral region all five lch5 neurons, v'ch1 and v'pda show expression. **C.** In the ventral region vchA and vchB both show expression. **D.** Schematic representation of the DS78 staining pattern. (A) Scale bar 0.1mm. (B-C) Scale bar 0.01mm.

DS97

Pattern of expression

Expression first appears in the sensory nervous system in a small lateral cluster of cells belonging to lch5, which is faintly labelled (Figure 3.22 B, asterisks). At later stages there is strong expression in all cells of the lateral chordotonal organ, including the non-neuronal support cells (Figure 3.22 D, E). Only one other sensory neuron is stained in abdominal segments. A single, extremely dorsal neuron is labelled, which from its position and morphology can only be desC or desD (Figure 3.22 F). Expression in this dorsal es neuron was only ever observed in A1-A3, however. In the procephalic sensory nervous system a paired anterior sensory structure within the antennomaxilliary complex is labelled (Figure 3.22 C). A small cluster of cells within the amnioserosa also show expression (Figure 3.22 B, arrowheads) and these cells may account for the collection of somewhat square cells staining at the dorsal-most point of the embryo in later stages, where both sides meet after dorsal closure (Figure 3.22 C). There is no CNS staining in this line.

Timing of expression

The first cells to begin expressing GFP in DS97 are a small cluster of cells in the head region of the embryo (Figure 3.22 A, arrow) which susbequently form one of the procephalic sense organs. These cells are apparent from stage 12 onwards. Staining begins to be detected within the amnioserosa at about stage 13 and expression also appears in the sensory nervous system at this time.

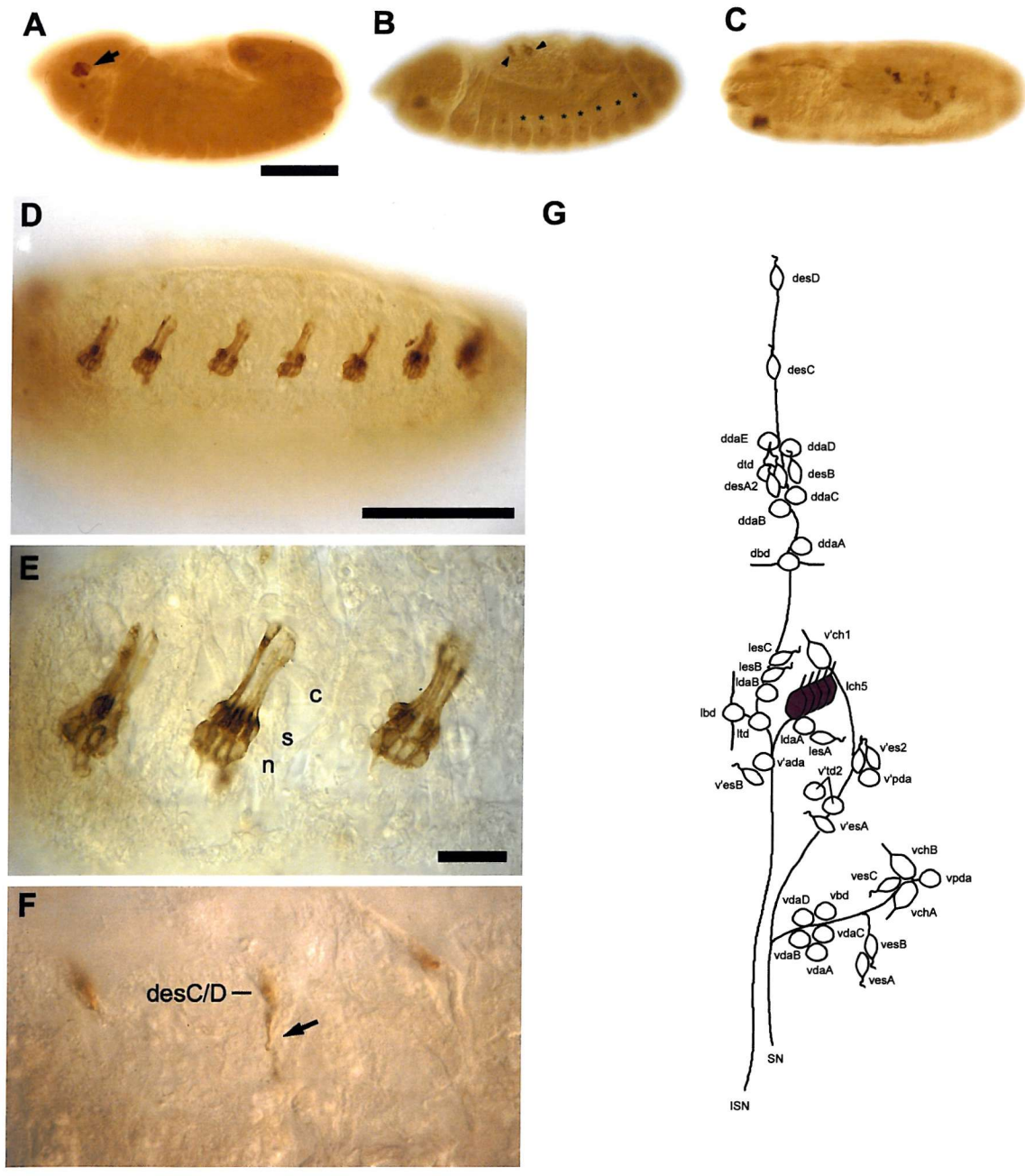


Figure 3.22 DS97 Expression Pattern. A. Stage 12 embryo showing expression in a cluster of cells in the presumptive head region (arrow). **B.** Lateral view of a stage 13 embryo showing staining of amnioserosa cells. **C.** Dorsal view showing expression in a number of cells at the dorsal edge of the embryo. **D.** Lateral view of a stage 15 embryo showing expression in the lateral chordotonal organs. **E.** Within each lateral chordotonal organ, the neuron (n), scolopale cell (s) and cap cell (c) are labelled. **F.** A single dorsal es neuron, which could be either desC or desD, shows expression in A1-A3. The axon of this neuron is also labelled (arrow). **G.** Schematic representation of the DS97 staining pattern. (A-C) Scale bar 0.1mm. (D) Scale bar 0.1mm. (E-F) Scale bar 0.01mm.

3.5 Discussion

The GAL4 system provides a convenient means of misexpressing a variety of different genes in discrete subset of neurons, simply by crossing a suitable P[GAL4] line with each different UAS-gene of interest line to be tested. In order to identify suitable P[GAL4] drivers I screened an existing collection of P[GAL4] lines that had previously been screened during larval and pupal development. As these lines had been retained on the basis of expression in sensory neurons, this approach had the advantage that all lines were already known to label sensory neurons. However, as I wished to determine whether patterning genes have a function in determining sensory neuron projections, it was important to identify lines that would enable a gene to be misexpressed prior to sensory axon ingrowth. I therefore screened these lines embryonically to determine the onset of GAL4 activity for each line.

3.5.1 Late onset of sensory nervous system expression

Of the 41 enhancer-trap lines screened during this project, 21 lines showed expression in the sensory nervous system during embryonic development. The majority of these lines however only began to express GFP at around stage 15-16, after the nervous system was fully formed. Very few of the lines showed expression at earlier stages and none showed expression in sense organ precursors. While expression at prior stages in sense organ development was not a prerequisite, as the only requirement was that sensory neurons showed expression prior to sensory axon ingrowth, in practice the slight lag in reporter gene activation after GAL4 becomes activated might be critical because sensory axon ingrowth occurs shortly after the sensory neuron is produced (Kolodziej et al., 1995). It would therefore have been advantageous for GAL4 activity to be present in a lineage prior to the birth of the sensory neuron.

The lack of P[GAL4] lines that are expressed throughout neuronal development could reflect a division in gene expression between precursor cells and neurons. Many genes expressed in sense organ precursors, such as the proneural genes or *daughterless* are not expressed in their progeny. This would support the notion of a boundary between gene expression in the developing nervous system and neuronal gene expression after the terminal mitosis in each lineage. A few genes, such as *cut*, have been identified which are expressed at all stages of neuronal development, from precursor to neuron. It is clear however that of the genes that have so far been identified as being expressed in sensory neurons or their precursors, by far the majority are expressed in either neurons or precursors and not both.

The existence of enhancer-trap line such as A37 (Ghysen and O'Kane, 1989) which are expressed both during sense organ precursor formation and in the fully formed nervous system would suggest that there is no reason per se why enhancer-trap lines cannot label both precursor cells and neurons. However A37 is a P[lacZ] enhancer-trap element. A far lower frequency of transposition has been reported for the GAL4 enhancer-trap technique than for lacZ enhancer-traps (Gustafson and Boulianne, 1996). If genes expressed throughout nervous system development truly are rare, then the lower insertional frequency of GAL4 enhancer-traps may be significant in the identification of lines expressed from early stages of sensory neuron development onwards. Techniques for the conversion of transposable elements have now been reported (Sepp and Auld, 1999) and perhaps in future the conversion of P[lacZ] enhancer-trap lines already known to label sensory neurons, such as the md-specific lacZ line (Bier et al., 1989) or A37 itself, to P[GAL4] enhancer-trap lines might prove useful.

In addition, as activation of the enhancer-trap element is detected indirectly in P[GAL4] enhancer-trap lines via a secondary reporter gene such as GFP, the time interval between initial activation of gene expression and first detection of reporter gene activity may be substantially greater than with P[lacZ] enhancer-trap lines. Such a delay could prove significant when visualising short-lived cells such as neuronal precursors as the time taken to achieve a detectable GFP signal could be longer than the time available prior to neuronal precursor division. Furthermore the number of GFP molecules required to produce a detectable signal is likely to be around 500, 000 molecules per cell (C. O'Kane, personal communication). The lack of any observed GFP expression in a particular cell-type does not therefore necessarily mean that GAL4 expression has not been activated, just that the levels of GAL4 protein produced are insufficient to promote a detectable level of GFP expression. However, the ability to visualise neuronal precursors in other P[GAL4] lines, such as the daughterless-GAL4 line, makes this explanation unlikely. Neither is it likely that the lag in the ability to visualise GAL4 activity could account for the late onset of sensory nervous system expression in the majority of the lines examined. Expression began to be detected at stage 12-13 in a number of lines, including DS43, DS58, DS69 and DS97, suggesting there is no a priori reason why P[GAL4] lines cannot show early onset of expression within sensory neurons.

3.5.2 Bias in the screen

Another explanation for the relatively late onset of expression in the majority of P[GAL4] lines examined is that, by starting with an existing collection of lines already known to be expressed in sensory neurons at later stages in development, I have biased the screen towards the recovery of lines that show expression in mature neurons. As the lines in the collection were originally selected on the basis of expression in sensory neurons during pupal stages and late larval stages, GAL4 expression in these lines is perhaps more likely to be under the control of enhancers required for neuronal function or maintenance of gene expression, rather than those that are active during neuronal development or sensory axon ingrowth. It is therefore perhaps not surprising that so few lines were recovered showing expression at early stages of embryonic development.

Many of the lines identified in this screen do not begin to be expressed until the final stages of embryonic development, once the nervous system is fully formed and sensory neurons have long established specific patterns of connections with the central nervous system. In some cases, such as DS74, expression only become detectable just prior to hatching and could perhaps be more truly considered to represent larval rather than embryonic expression. The extremely late onset of GFP expression in some lines is also the explanation for the difference between the number of lines selected for further study on the basis of GFP expression in live embryos and the number showing expression in sensory neurons when examined using antibody staining. A relatively small number of embryos ($n \geq 20$) were screened initially and GFP can be detected fluorescently in embryos right up to hatching, leading to the selection of some lines for further study on the basis of GFP expression in sensory neurons at late embryonic stages. Antibody staining of embryos using conventional techniques is only effective up to stage 17, prior to cuticle formation and, if the onset of GFP expression in a particular line only occurs after cuticle formation, no expression will be detected using conventional immunohistochemistry. Unambiguous identification of many sensory neurons in live embryos at these late stages is difficult due to the waves of somatic muscle contraction that are occurring. As these lines were already determined to be of no use as misexpression vectors and do not truly reflect embryonic sensory nervous system expression, no further steps were taken to confirm the identity of the sensory neuron complement of those lines.

3.5.3 Most lines do not exclusively reveal sensory neurons

The majority of lines revealed both central and peripheral neurons. This presents a number of problems. Firstly, when a GAL4 line is being used both to visualise a specific subset of neurons and to misexpress a particular gene in those cells, staining within the CNS may physically obscure the central projections of sensory neurons. Such expression may also make it difficult to determine whether particular axon branches belong to an ingrowing sensory neuron or form part of the projections of central neurons in the same area. This could be surmounted by independently visualising sensory neuron morphology (see section 3.5.8 below).

Secondly, when examining neuronal defects caused by gene misexpression it is essential that one can be confident that any observed defects are the consequence of ectopic expression in the targeted structure, and not the result of ectopic expression in additional structures. For examining potential disruptions of sensory projections in the CNS it is especially important to be certain that the defects could not be caused by ectopic expression within the CNS itself. If a gene is being misexpressed both in subsets of peripheral and central neurons, any central projection defect could therefore merely be the result of a CNS defect that disrupts the substrate on which the sensory neurons navigate.

It is likely also that during the process of establishing a specific pattern of central projection sensory neurons receive cues from their CNS target area. Target recognition by *Drosophila* motorneurons appears to rely on unique features of individual target muscles (Chiba et al., 1993; Sink and Whitington, 1991) and similar mechanisms probably operate during synaptic recognition by sensory neurons. Any changes in sensory projection resulting from ectopic expression using these P[GAL4] lines could not simply be attributed to changes in gene expression within sensory neurons, as some central neurons would also show ectopic expression, which might exert a non-cell autonomous effect on axonal arborisation pattern.

Early expression within the epidermis could lead to complications with respect to the misexpression of patterning genes, as it would then be difficult to determine whether any subsequent defects were a secondary consequence of disruption of patterning information in the epidermis rather than a direct result of ectopic expression in sensory neurons. However all lines showing significant epidermal staining do not begin to be expressed until after the sense organ precursors have delaminated. Epidermal expression therefore primarily presents a problem in the identification of sensory neurons present. Such expression would also obscure

the visualisation of neuronal morphology and axon trajectory in the periphery after misexpression.

3.5.4 Most P[GAL4] lines reveal chordotonal neurons

The results of the screen showed a large number of lines revealing chordotonal neurons. Of the nineteen lines where the pattern of sensory nervous system was clearly visible and not concealed by epidermal staining, all but one incorporated chordotonal neurons and more than 40% of these exclusively labelled chordotonal neurons. In addition, very few lines contained external sensory neurons. Only three lines showed any expression in external sensory neurons and none contained external sensory neurons alone. A similar bias towards the labelling of chordotonal neurons and against the labelling of external sensory neurons was observed during the original screen of 1400 P[GAL4] lines performed by Smith and Shepherd (D. Shepherd, personal communication). As this screen for embryonic PNS expression was not a de novo screen, and is subsequently constrained to an extent by the classes of sensory neuron expression already observed and selected for in the original screen, it is perhaps unsurprising that the same classes of sensory neurons are over- and under-represented in the embryonic screen. Most adult sensory neurons are produced during metamorphosis however and are not present in the embryonic nervous system. As there is no direct correspondence between the neurons observed in the pupal CNS and the majority of those present prior to metamorphosis, the same classes of sensory neuron would not necessarily be expected to be present in the embryo.

Such an extremely high incidence of chordotonal neuron expression, in particular of lch5 staining, does also raise the possibility that such expression could reflect some kind of background GAL4 activity in the chordotonal organs. It is unclear how many other GAL4 lines show expression in the lch5 organ as a matter of course. A large collection of lines with PNS expression also exists on flyview (http://flyview.uni-muenster.de/) and from the images available it would seem like a significant number of these also show expression in a lateral position consistent with lch5, although it is impossible to be sure at the resolution available on the website.

3.5.5 Can the observed patterns of embryonic sensory nervous system expression be subdivided by location?

Those lines showing expression in multidendritic and external sensory neurons, which occur throughout each hemisegment, were examined to determine if there was any subdivision of expression pattern according to location. This is shown in Table 3.4. Several lines do reveal neurons in ventral and lateral but not dorsal regions of the embryo, while one line covers dorsal and lateral but not ventral regions. No lines are restricted to just one area however and in general staining occurs throughout the periphery, suggesting that certainly as far as P[GAL4] enhancer-trap expression patterns are concerned there is no underlying subdivision of the sensory nervous system into spatial domains.

Table 3.4 Comparison of type of neuron present, peripheral location and proneural gene dependency

DS Line	Proneural gene dependency	Type of neuron present	Neuronal locations
DS04	atonal	ch. only	n.a.
DS20	atonal	ch. only	n.a.
DS24	atonal and AS-C	ch. and es.	all regions
DS27	atonal and AS-C	ch. and md.	ventral and lateral regions
DS35	atonal and AS-C	ch. and md.	ventral and lateral regions
DS44	atonal	ch. only	n.a.
DS46	AS-C and amos	md only	dorsal, lateral, and v' regions
DS47	atonal, AS-C and amos	ch, md and es	all regions
DS54	atonal (and amos)	ch (and md)	n.a.
DS59	atonal	ch. only	n.a.
DS60	atonal (and AS-C)	ch. (and md)	n.a.
DS72	atonal and amos and AS-C	ch and md (and es)	all regions
DS73	atonal	ch. only	n.a.
DS74	atonal	ch. only	n.a.
DS77	atonal	ch. only	n.a.
DS78	atonal and AS-C	ch and md	ventral and lateral regions
DS97	atonal and AS-C	ch and es	dorsal and lateral regions

3.5.6 Do boundaries of enhancer-trap expression patterns reflect any functional subdivision of the classes of neurons present?

Multidendritic neurons can be divided into three different subclasses based on their morphology, and these different types of multidendritic neuron may serve different sensory functions. While several lines contain subsets of multidendritic neurons, there is no subdivision according to different subclasses, as summarised in Table 3.5. Multidendritic neurons can also be divided into different groups based on their central projection pattern, and these different sensory arborisation domains may again reflect functional differences between the different types of multidendritic neurons (Merritt and Whitingdon, 1995). Different subsets of multidendritic neurons present in different P[GAL4] enhancer-trap lines show no correlation with central projection pattern however.

Table 3.5 Subclasses of multidendritic neuron, proneural gene dependency and projection pattern

P[GAL4] Enhancer-trap line	Class of md neuron present	md proneural gene dependency	Projection pattern (heterogeneous, md-da, or both)
DS27	md-bd, md-td, md-da	AS-C and atonal	heterogeneous and md-da
DS35	md-bd, md-td, md-da	AS-C and atonal	heterogeneous and md-da
DS46	md-bd, md-da	AS-C and amos	heterogeneous and md-da
DS72	md-bd, md-da	amos, atonal, and AS-C	heterogeneous and md-da

A further possibility is that boundaries of enhancer-trap expression patterns could coincide with boundaries of proneural gene dependence. While chordotonal neurons and external sensory neurons are dependent on atonal and the genes of the AS-C respectively, some multidendritic neurons are specified by atonal, some by AS-C and the remainder by an atonal-related proneural gene, amos. As many lines reveal subsets of neurons, a possible relationship could exist between the subsets of neurons present and the proneural gene responsible for their specification. As shown in Table 3.4 and Table 3.5, no correspondence is apparent between class of proneural gene and the subset of multidendritic neurons present, and there do not appear to be any obvious factors relating the subsets of neurons revealed by each line.

3.5.7 Variability in enhancer-trap staining patterns

Within each line, there is some degree of variability in the number of neurons stained from individual to individual and between different segments in the same embryo. This type of fluctuation in staining pattern is not restricted to the GAL4 lines in this screen. For example, the MARCM project (see Chapter 6) has provided ample opportunity to analyse the elav-GAL4 enhancer-trap expression pattern, which should label all neurons. This line also exhibits great variability, in both the number of neurons stained between individuals and the number of neurons stained between segments. This is particularly obvious for lch5, where there is variation not just in the number of lch5 neurons expressing GFP between segments but also in which lch5 neurons are expressing. This variability may partially stem from the heterozygous state of the elav-GAL4 element. Elav-GAL4 may not recapitulate the wild-type pattern of Elav protein expression because, when present in one copy only, the construct is not able to drive a sufficiently high level of reporter gene expression to reveal all neurons. As all lines have been screened as heterozygotes, the variability in the number of neurons visualised may therefore signify that the endogenous enhancer is unable to reliably drive sufficiently high levels of reporter gene expression in all neurons in which it is active, meaning that certain neurons do not always accumulate enough GAL4 to be positively stained.

Epigenetic factors may also influence the penetrance of a particular P[GAL4] line. Position effect variegation occurs when a gene is closely juxtaposed to heterochromatin, and results in mosaic silencing of gene expression. This variegation is thought to be a consequence of cell-cell variation in the extent of heterochromatic structures, such that in some cells a gene will be incorporated into heterochromatin but not in others, leading to mosaic gene inactivation (Tartoff et al., 1989). A recent study has shown that position effect variegation is extremely sensitive to transcription factor levels and that binding of transcription factors can counteract heterochromatic gene silencing (Ahmad and Henikoff, 2001). This indicates that heterochromatin probably affects the availability of transcription factor binding sites and that low levels of transcription factors result in enhancers essential for gene transcription being bound in fewer cells, resulting in a higher frequency of heterochromatic gene silencing. Insertion of P[GAL4] lines close to heterochromatic regions of the chromosome could therefore provide an explanation for some of the variability in enhancer-trap expression that has been observed during this screen, with weakly expressed P[GAL4] lines juxtaposed to heterochromatin predicted to be more susceptible to position effect variegation than strongly expressed lines.

3.5.8 Visualisation techniques

Although GFP can be visualised directly using epifluorescence I decided to visualise the sensory neurons revealed by each line using antibodies against the GFP reporter gene. I chose to use a tau-GFP fusion protein as a reporter because the inclusion of the tau coding sequence targets the GFP to microtubules (Brand and Dormand, 1995). The entire morphology of the cells expressing GAL4 can thus be visualised, facilitating their identification. In addition, GFP can be directly visualised in live embryos, enabling rapid selection of lines for further study. The ability to visualise the structure of sensory neurons, including axons and dendrites has indeed proved a great aid to identification, both in ascertaining whether a peripheral cell is neuronal or not and in determining to which class a sensory neuron belongs. Axons were often more clearly visible with epifluorescence as opposed to antibodies, because they can be examined at later stages when GFP has had a longer time to accumulate. The large amount of muscle contraction occurring precluded accurate identification of sensory neurons however, necessitating immunohistochemical detection of GFP expression.

As mentioned above, while GAL4 may provide a convenient means of achieving ectopic expression it might sometimes prove advantageous to be able to reveal neuronal morphology by independent means. Intracellular injection of lucifer yellow and lipophyllic dialkylcarbocyanin fluorescent dyes such as DiI have both been used successfully to examine sensory projections in the *Drosophila* embryo (Merritt and Whitington, 1995). Such techniques enable the axonal projections of individual neurons to be visualised.

3.5.9 Problems of tau as a reporter gene

Tau-GFP is one of the most widely used neuronal reporter genes (Phelps and Brand, 1998). By targeting GFP to microtubules, the neuron's own axonal transport system is used to distribute the protein throughout the cell (Merritt and Whitington, 1995). During the course of this thesis however, studies from our laboratory showed that tau reporter genes cause severe defects in sensory neurons and neuronal degeneration (Williams et al., 2000). Such defects do not appear to affect sensory neuron development however, as axon growth and target region innervation appear normal. During embryonic development, tau-based reporter genes such as tau-GFP have been routinely used to visualise neurons with no published detrimental effect on nervous system development, supporting the notion that neuronal development is unaffected and that defects do not appear until later life, consistent with the features of human

tauopathies. Therefore, while tau-based reporters should clearly be avoided in future, the use of tau-GFP during this embryonic screen would not affect the results obtained.

More recently however, problems in embryonic motorneuron pathfinding and occasional loss of motorneurons have been observed as a result of tau-GFP expression (Andreas Prokop, personal communication). While pathfinding defects are of no direct concern, as the main purpose of this screen was to determine the identity of the sensory neurons revealed by each P[GAL4] line, possible neuron loss could affect the results of the screen. However, occasional neuron loss could not account for the degree of variability seen during this screen and, as the general staining pattern of each line was consistent between embryos, I believe that the pattern of neuronal expression observed for each line is probably accurate. Any lines to be used as misexpression vectors could quickly be re-screened using a different reporter gene, such as CD8-GFP (Lee and Luo, 1999). This fusion protein also enables neuronal morphology to be revealed, by targeting GFP to the plasma membrane, but was not available at the time the screen was undertaken.

Chapter 4. Ectopic Expression using the Gal4 Enhancer-trap technique

4.1 Aim

Ectopic expression can be a powerful means of testing a gene's role in determining cellular identity during development. Misexpression of candidate genes previously identified in an antibody screen throughout the entire nervous system provides a means of testing whether these genes can influence nervous system development. The aim of the work presented in this chapter is therefore to provide a proof of principle and show that the candidate genes identified in the antibody screen can affect nervous system development.

4.2 Introduction

The analysis of gene expression within the sensory nervous system that was presented in Chapter Two identified three candidate segmentation genes that may play a role in the cell fate specification of individual sensory neurons: *runt*, *engrailed*, and *armadillo*. One means of testing whether these genes are likely to be involved in nervous system development would be to misexpress the candidate genes in all neurons and ascertain whether nervous system development is affected. Those genes that disrupted nervous system development when expressed in all neurons would be strong candidates for playing a role in the development of an ordered nervous system.

Pan-neuronal expression of candidate gene can be accomplished using the GAL4 enhancer-trap technique (reviewed in Chapter Three). The C155-GAL4 enhancer-trap line (hereafter known as elav-GAL4) is expressed is the same temporal and spatial pattern as the *elav* (eyes lethal abnormal vision) gene, a pan-neuronally expressed RNA binding protein which regulates the differential splicing of the neural specific forms of a number of genes, including *neuroglian* (Koushika et al., 1996). The elav-GAL4 line therefore provides a convenient means of misexpressing any gene that is coupled to the UAS-promoter in all neurons. As both *armadillo* and *engrailed* are readily available as UAS-responder lines, their effect on nervous system development when expressed pan-neuronally can be tested.

4.3 Materials and Methods

4.3.1 Fly Stocks

All flystocks were obtained from the Bloomington stock centre (Indiana, USA) and maintained on standard Drosophila media (Appendix A) at room temperature. Virgin females homozygous for the elav-GAL4 enhancer-trap insert were crossed with either males containing a homozygous UAS-*armadillo* construct on the third chromosome, or males containing a heterozygous UAS-*engrailed* insert on the TM6 balancer chromosome. Stocks of Oregon R (OreR) Wild-type flies were used to show the normal pattern of sensory nervous system development.

4.3.2 Examination of embryos

Embryos were collected on apple-juice agar plates (Appendix A). Each cross was left to lay overnight at 25^0C to yield embryos at a variety of developmental stages.

4.3.3 Immunohistochemistry

Immunohistochemistry was carried out as described in Section 2.2 and the sensory nervous system was visualised using the Mab22c10 antibody (Developmental Studies Hybridoma bank, USA) at a dilution of 1:250. For double staining of elav-GAL4 x UAS-*engrailed* embryos the anti-Engrailed antibody was used at 1:5 and the Mab22c10 antibody was used at 1:250.

4.3.4 Microscopy

Embryos were subsequently examined using a Zeiss Axiophot II microscope equipped with a digital camera and images were captured using Metamorph software. All figures were created in Adobe Photoshop v5.0.

4.4 Results

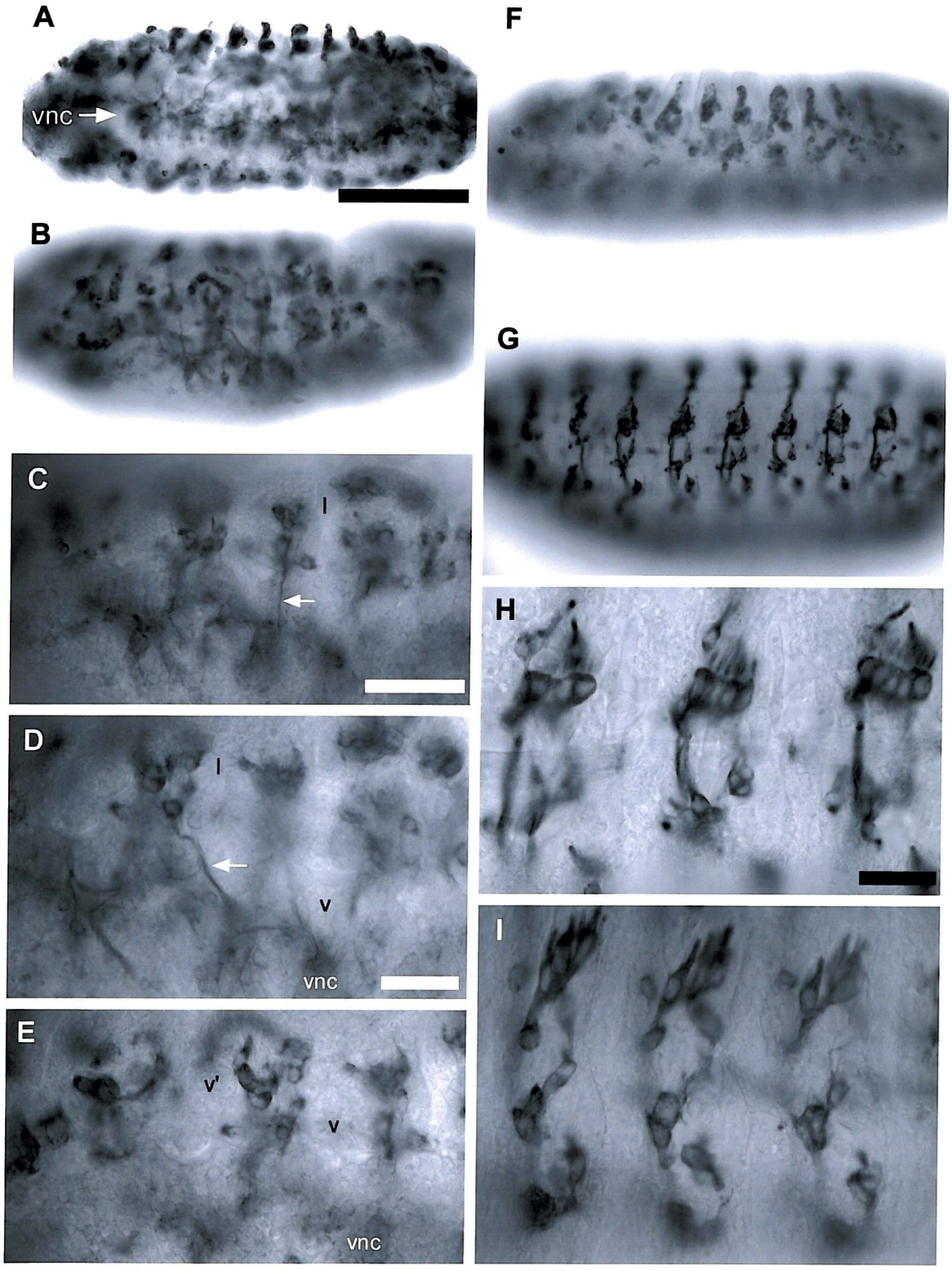
4.4.1 Pan-neural expression of *armadillo*

The UAS-*armadillo* transgene was expressed under the control of the pan-neuronal elav-GAL4 enhancer-trap line in order to activate *armadillo* expression in all embryonic neurons. To examine the effects of ectopic *armadillo* expression on sensory nervous system development, the Mab22c10 antibody, which labels all sensory neurons, was used to visualise the embryonic sensory nervous system (Figure 4.1). Pan-neural expression of *armadillo* results in sensory nervous system development being totally disrupted. There is little semblance of a highly ordered and segmentally repeated sensory nervous system evident, with both the distribution of sensory neurons and the formation of axon pathways being severely affected (compare Figure 4.1 G to Figure 4.1 A, and B). In many segments it is difficult to distinguish the peripheral nerve pathways, and in those segments where they can be made out the stereotyped pattern of axon pathways is severely disorganised, with failures in axon fasciculation and pathway selection. For example, the axons in Figure 4.1 D which would normally form part of the SN, no longer fasciculate together. A third discrete axon bundle may also normally form part of the SN or may alternatively form the ISN but the degree of disruption within the sensory nervous system prevents accurate identification of individual neurons. In wild type embryos all ventro-lateral neurons project along branches of the SN but in Figure 4.1 C a ventro-lateral neuron extends an axon towards the ISN.

While the gross distribution of sensory neurons into dorsal, lateral, ventro-lateral (v') and ventral groups neuronal cell bodies is still roughly preserved, the sensory neurons that comprise each cluster bear little resemblance to the characteristic pattern of sensory neurons in the wild type embryo. Some clusters appear to contain too many neurons (Figure 4.1 E compared to Figure 4.1 I) while some contain too few (Figure 4.1 C compared to Figure 4.1 H). In addition, the cell bodies are frequently tightly clustered and there are no obvious differences in neuronal morphology between individual neurons. For example the lateral chordotonal organ, lch5, which is easily distinguished in wild type embryos (Figure 4.1 H) cannot be identified in elav-GAL4 x UAS-*armadillo* embryos (Figure 4.1 C).

The extreme degree of sensory nervous system disruption observed in elav-GAL4 x UAS-*armadillo* embryos suggested that the development of the whole embryo might have been affected. Much of the observed disruption to sensory nervous system development might therefore be a consequence of a more general defect in embryonic development. While

Figure 4.1 Ectopic expression of *armadillo* in all neurons. A-F, elav-GAL4 x UAS-armadillo embryos. G-I, OreR embryos. **A.** Ventral view showing disruption of ventral nerve cord formation. **B.** Lateral view showing disruption of sensory nervous system formation. **C.** The lateral region is totally disrupted. Arrow indicates peripheral nerve. **D.** The ventro-lateral region is highly disorganised, although peripheral nerves are still visible in some segments. **E.** Clusters of cells are still present in the approximate positions of the ventral and ventro-lateral neurons. **F.** A dorsal view showing that cells are present in the approximate location of the dorsal cluster, but the morphology of the cluster is grossly abnormal. **G.** Lateral view showing the sensory nervous system in wild-type embryos. **H.** Showing the lateral region in greater detail. **I.** Showing the ventral and ventro-lateral region in greater detail. (A-B, F-G) Scale bar 0.1mm. (C) Scale bar 0.03mm. (D-E, H-I) Scale bar 15μm.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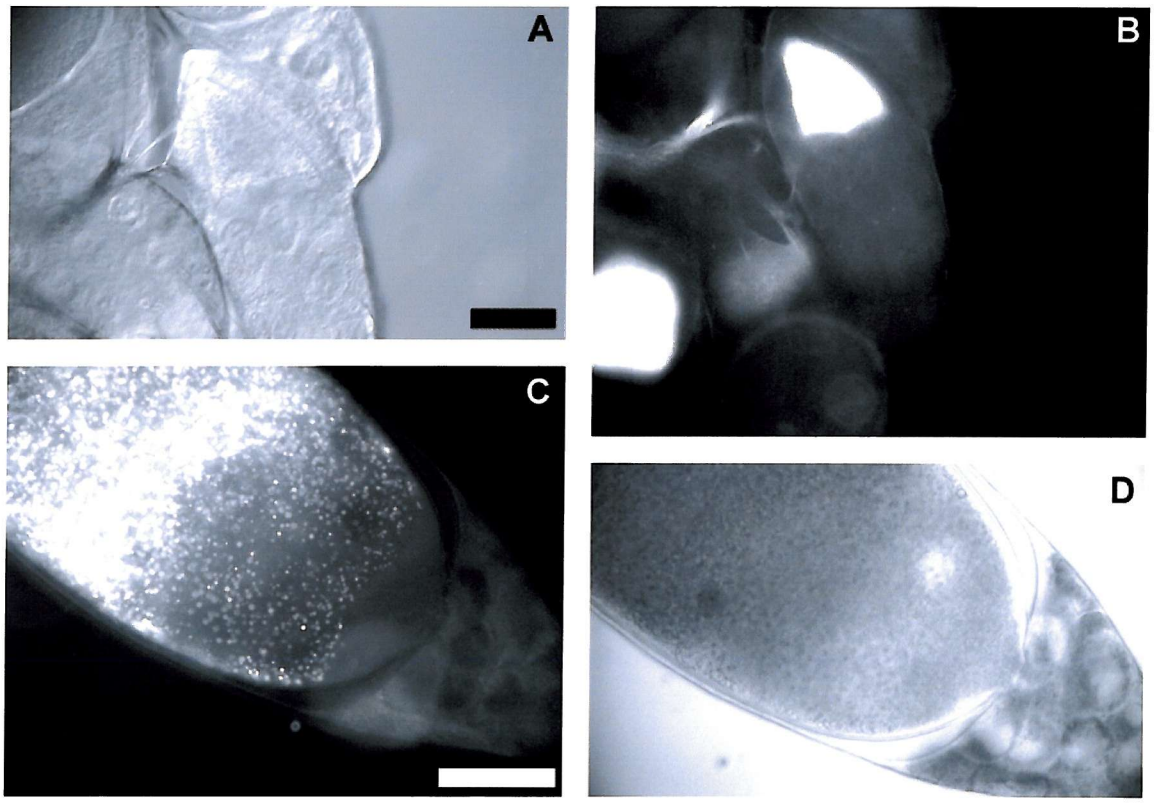


Figure 4.2 Elav-GAL4 x UAS-GFP expression in oocytes. A. Brightfield view of egg chambers at an early stage in oogenesis. **B.** GFP expression is visible throughout the oocyte **C.** Brightfiled view of an egg chamber at a later stage in oogenesis. D. GFP expression is still present in the oocyte cytoplasm. (A-B) Scale bar 0.1mm. (C-D) Scale bar 0.05mm.

armadillo plays several roles during embryonic development the use of the elav-GAL4 enhancer-trap line as a means of misexpressing *armadillo* in all neurons should ensure that *armadillo* overexpression is confined to the nervous system. To investigate the specificity of the elav-GAL4 enhancer-trap line, its expression was examined throughout development using GFP as a reporter gene. While expression was not detected at any earlier stage in embryonic development, there is significant expression in oocytes (Figure 4.2). As *armadillo* is required during cellularisation for the formation of adherens junctions establishment of epithelial polarity (Cox et al., 1996; Muller and Wieschaus, 1996) the *armadillo* expression that would be ectopically activated in oocytes may be sufficient to perturb this process and may be responsible for the gross nature of the defects to sensory nervous system organisation observed in elav-GAL4 x UAS-*armadillo* embryos.

4.4.2 Pan-neural expression of *engrailed*

The UAS-*engrailed* transgene was expressed under the control of the pan-neuronal elav-GAL4 enhancer-trap line in order to activate *engrailed* expression in all embryonic neurons, and the embryonic sensory nervous system was then visualised using Mab22c10. Pan-neural expression of *engrailed* causes severe disruptions in sensory nervous system organisation (Figure 4.3). There are two main defects. First, peripheral pathfinding is affected. In wild type embryos the peripheral pathways are highly stereotyped and in A1-A7, all sensory axons fasciculate with either the ISN or the SN and enter the CNS through their segment of origin. However, in elav-GAL4 x UAS-*engrailed* embryos axons from the dorsal cluster misproject into the neighbouring segment and fasciculate with the ISN to enter the CNS in the anterior segment to their segment of origin (Figure 4.3 E, arrow). There are also slight defects in the formation of the SN nerve pathways and occasionally the segmental nerve is missing (Figure 4.3 K, L). In this segment the intersegmental nerve also projects at a more internal level than normal (Figure 4.3 K, L). When the segmental nerve is not present, some ventro-lateral neurons are observed to initially extend an axon in the appropriate direction (Figure 4.3H, arrowhead), which then turns dorsally until it reaches the lch5 neurons (Figure 4.3 H, white arrow). It is not clear whether axon extension terminates here or whether the axon continues to join the ISN. Such defects are incompletely penetrant and are not present in all segments.

Second, there is often a reduction in the number of lateral chordotonal neurons in many segments (Figure 4.3 I, white arrow), and in some segments the entire lateral pentascolopale is missing (Figure 4.3 E, G, H). In addition, the positioning of the vchA chordotonal neuron is altered in some segments, where vchA is found in parallel with vchB (Figure 4.3 J). The

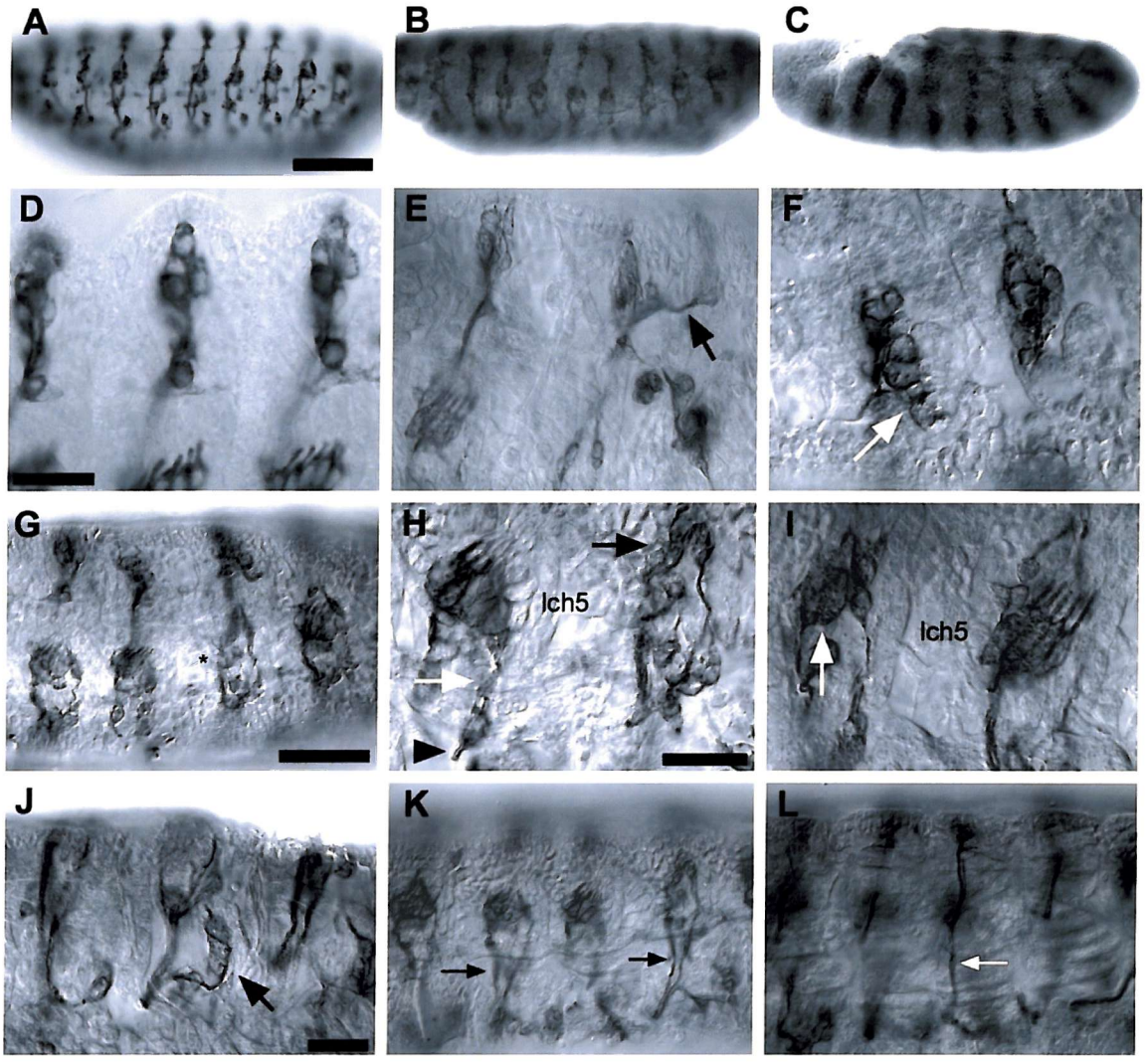


Figure 4.3 Ectopic expression of engrailed in all neurons. A, D OreR. B-C, D-L elav-GAL4 x UAS-engrailed. **A.** Lateral view of sensory nervous system in wild type embryo. **B.** Lateral view of elav-GAL4 x UAS-engrailed embryo. **C.** Engrailed segment polarity gene expression is normal in elav-GAL4 x UAS-engrailed embryos. **D.** Dorsal cluster in wild type embryos. **E.** Shows the dorsal cluster misprojecting into the anterior segment. **F.** Cells are present below the level of dbd (arrow). **G.** The lch5 organ is missing (asterisk). **H.** Shows the absence of the lch5 organ in greater detail. **I.** There is a reduction in the number of lch5 neurons. Only 3 ch neurons are found in the lch5 organ position. **J.** The vch A neuron projects dorsally rather than ventrally. **K.** Showing the normal location of peripheral nerves and the presence of both the ISN and the SN in the two segments indicated by arrows. **L.** The same embryo in a different view. In the middle segment only one nerve has formed and is located much more internally than normal. (A-C) Scale bar 0.1mm. (D-F) Scale bar 0.03mm. (G) Scale bar 0.03mm. (H-I) Scale bar 0.01mm. (J-L) Scale bar 15μm

presence of additional cells below dbd in one segment is suggestive of an increase in cell number in the dorsal cluster (Figure 4.3 F) although the compactness of the dorsal cluster makes exact cell number difficult to determine.

Double-staining of elav-GAL4 x UAS-*engrailed* embryos for Engrailed and Mab22c10 showed that while Engrailed is expressed in the majority of sensory neurons, elav-GAL4 does not drive Engrailed expression in all neurons (Figure 4.3 F-L). Expression of Engrailed during germ band extension is normal, indicating that pattern formation is not affected (Figure 4.3 B).

4.5 Discussion

The misexpression of candidate genes in all neurons was designed to provide a crude test to show whether these genes could influence nervous system development and are therefore likely to be involved in specifying cell fate in the sensory nervous system. While pan-neural expression of both *engrailed* and *armadillo* caused severe defects in sensory nervous system organisation, such experiments provide little information as to what role these genes may play in sensory nervous system development. Nevertheless they demonstrate that these genes can affect neuronal development, thus providing a 'proof of principle' and accomplishing their aim.

4.5.1 Do the observed defects in sensory nervous system organisation result from misexpression in sensory neurons?

The complete disruption of nervous system development observed in elav-GAL4 x UAS-*armadillo* embryos suggested that the development of the whole embryo in addition to the development of the nervous system may have been affected. As the elav-GAL4 line has been shown to activate gene expression from the UAS-promoter in oocytes and there is an early requirement for *armadillo* during cellularisation (Cox et al., 1996; Muller and Wieschaus, 1996) it is likely that the severity of the sensory nervous system defects that were observed may partly reflect the overexpression of *armadillo* during cellularisation, as the maternally produced *armadillo* would probably still be present at this stage. During cellularisation, *armadillo* is required for the formation of both a basal and an apical junction between cells (Cox et al., 1996; Muller and Wieschaus, 1996). The basal junction forms first and is required for cellularisation and the apical junction is required for polarity and maintenance of the epithelium. As the relocalisation of Armadillo from the basal to the apical junction is tightly regulated (Hunter and Wieschaus, 2000), overexpression of Armadillo could interfere with the stabilisation of the basal junction or cause the apical junction to form too early, which may affect gastrulation, as absence of the apical junction is critical for the cell shape changes in the ventral furrow (Hunter and Wieschaus, 2000).

The *engrailed* gene is first expressed during germ band extension and shows a normal pattern of segment polarity expression, indicating that epidermal patterning has not been affected. The defects in sensory nervous system development observed in elav-GAL4 x UAS-*engrailed* embryos are therefore likely to result from ectopic *engrailed* expression in the nervous system.

4.5.2 Is ectopic expression a reliable indicator of a role in sensory neuron development?

When a protein that is not normally present in a cell or is present in small quantities is produced in large amounts, it is conceivable that normal cellular development could be affected in a non-specific manner. Cellular functions could be passively disrupted through the physical presence of large amounts of a protein, even if that protein was non-functional in that cell type. Cellular systems could become congested, diverting cellular resources away from the production and processing of proteins native to the cells and essential for its normal development.

The ectopic expression of reporter genes that are not natively expressed, such as GFP and lacZ, using the P[GAL4] system has no discernible effect on cellular development however, suggesting that it is unlikely that the amount of protein produced when a gene is overexpressed using the GAL4 enhancer-trap system would be sufficient to produce a detectable disruption of cellular function through sheer physical abundance. In addition the elav-GAL4 driver is a comparatively weak GAL4 driver and, when used to express GFP, is not able to drive a sufficiently high level of reporter gene expression to reveal all embryonic sensory neurons (L. Block, unpublished observations).

4.5.3 Overexpression of *engrailed*

Elav-GAL4 x UAS-*engrailed* embryos displayed a number of pathfinding defects and both gain and loss of neurons. These defects were not completely penetrant and this is partly due to the significant degree of variability that has been observed for the elav-GAL4 expression pattern (L. Block, unpublished observations). Double-staining of elav-GAL4 x UAS-*engrailed* embryos for both Engrailed and Mab22c10 confirmed that Engrailed is not being expressed in all sensory or central neurons. Not all Engrailed-expressing neurons show defects, however. This could indicate that not all neurons are equally susceptible to ectopic *engrailed* expression and, if *engrailed* expression is not being driven at the same level in all neurons, that a threshold level of *engrailed* expression is required to alter sensory neuron development.

4.5.3.1 Peripheral pathfinding defects

The most obvious pathfinding defect caused by *engrailed* misexpression is the misprojection of the dorsal cluster into neighbouring segments. Such defects do not necessarily imply a specific role for *engrailed* in peripheral pathfinding. Many peripheral pathfinding mutants, such as *jaywalker* (Salzberg et al., 1994) and *astray* (Prokopenko et al., 2000; Salzberg et al., 1997), display similar defects, perhaps implying that this step in peripheral pathfinding is particularly sensitive to perturbation. These defects may merely reflect pathfinding defects in motorneurons as a result of ectopic *engrailed* expression that causes secondary defects in sensory neuron pathfinding as a result of the loss of guidance cues normally provided by motorneurons. If such defects are a direct consequence of Engrailed expression in sensory neurons then it would be interesting to determine if the sensory neuron that pioneers the axon pathway from the dorsal cluster (Hartenstein, 1988) is the *engrailed*-expressing dorsal cluster neuron.

Occasionally, the intersegmental nerve formed at a more internal level than normal and if engrailed overexpression disrupts motorneuron pathfinding, this too is likely to reflect a lack of guidance cues normally provided by the motorneurons.

4.5.3.2 Loss of chordotonal neurons

The overexpression of *engrailed* resulted in a reduction of lch5 chordotonal neurons in many segments, and occasionally, the complete loss of the lch5 chordotonal organ. As 3-4 of the lch5 neurons transiently express *engrailed* during normal development (Figure 2.5 B), the loss of chordotonal neurons may result from *engrailed* overexpression and downregulation of engrailed expression may be critical in these cells. Loss of chordotonal neurons cannot reflect a failure in chordotonal organ precursor recruitment as overexpression is only occurring in differentiated sensory neurons. The effects of *engrailed* overexpression on chordotonal neuron cell fate cannot easily be explained.

The severe defects in sensory nervous system formation cause by overexpression of *engrailed* show that *engrailed* can influence nervous system development. This provides further support for *engrailed* as a determinant of cell fate within the sensory nervous system.

Chapter 5. Laser Induced Activation of Gene Expression

5.1 Aim

The aim of the work presented in this chapter was to develop a technique that would enable gene expression to be targeted to individual sensory neurons. The potential of laser-induced heatshock as a means of activating gene expression in single cells was explored. By combining the basic principle of laser induced heatshock with a method of site specific recombination, gene expression could be constituitively activated in target cells. The viability of such a technique for reliably activating gene expression in targeted cells was examined.

5.2 Introduction

From the analysis of the patterns of gene expression within the peripheral nervous system presented in Chapter 2, several candidate genes have been identified that are expressed in subsets of sensory neurons and may contribute to the specification of neuronal identity. As sensory neurons make specific patterns of central projections according to cell type and peripheral location, I am particularly interested to determine whether these candidate genes play a role in specifying central projection pattern. Ectopic expression of two of these genes, *engrailed* and *armadillo*, has shown that they are capable of influencing sensory nervous system development (Chapter Four) but tells little about their normal role in sensory neuron development or their effect on axonal morphology in the CNS. One means of determining the role of these genes in controlling axonal trajectories would be to misexpress these genes in individual sensory neurons and assess what effect this has on the final pattern of sensory projections of that neuron.

In order to achieve this we needed to develop a technique that would enable us to target gene expression to single cells. A system had been described previously where a laser was used to induce heat shock in single cells in *Drosophila* that contain the *lacZ* reporter gene coupled to the *Drosophila* heat shock promoter, hsp70 (Halfon et al., 1997). The heat shock initiates transcription at the heat shock promoter and causes *lacZ* expression in those cells receiving the stimulus. This results in β-galactosidase activity within individually targeted cells.

Laser activation of gene expression in individually targeted cells would therefore seem a suitable system for the misexpression of genes of interest within specific sensory neurons. The flexibility of *Drosophila* as a transgenic organism means that many lines already exist

with a wide variety of genes coupled to the hsp70 promoter, and these constructs could also be activated in target cells by a laser. One drawback to this technique however is that the heat shock response is inherently transient, persisting for less than one hour (Sorger, 1991). Consequently the resulting expression of any gene coupled to the hsp70 promoter will be transient. We therefore intend to combine the basic principle of using a laser to induce gene expression in individual cells with a method of site specific recombination that will result in constitutive gene expression in targeted cells.

The FLP recombinase site specific recombination system was originally adapted from the *Saccharomyces cerevisiae* 2μ plasmid for use in *Drosophila* (Golic and Lindquist, 1989) and has since been used as a method of generating mosaics (Chou and Perrimon, 1992; Dang and Perrimon, 1992), for cell lineage analysis (Harrison and Perrimon, 1993; Ito et al., 1997), as a means of engineering chromosome rearrangement (Golic and Golic, 1996) and to mobilise DNA elements to specific sites in the genome (Golic et al., 1997). Initial studies placed the FLP recombinase gene under the control of the hsp70 promoter and, in response to heat shock, FLP caused the excision of the FLP recombination target (FRT) site flanked *white* gene, making cells mosaic for this marker (Golic and Lindquist, 1989). This methodology has subsequently been combined with the GAL4 enhancer-trap technique (Brand and Perrimon, 1993), previously outlined in Figure 3.1, as a method of examining clonally related cells in the *Drosophila* brain (Ito et al., 1997). This system uses site-specific recombination to switch genes on rather than off, by catalyzing the excision of transcriptional termination signals that block gene expression. In order to mark cells, heat shock induces the expression of the yeast transcriptional activator GAL4, which in turn activates expression of any transgene placed under the control of a GAL4-dependent promoter (UAS_G), in this instance the reporter gene, *tau*. This is shown in Figure 5.1. The inclusion of the GAL4-UAS system in the FLP-FRT site specific recombination method provides a flexible means of constituitively misexpressing any gene that is coupled to the UAS promoter in those cells in which heat shock is induced. By using a laser to provide the heat-shock stimulus it should be possible to target individual cells and constituitively activate gene expression in single neurons.

Before we can begin to use laser-activated gene expression to misexpress candidate genes in single identified neurons, its potential for providing a means of constituitively activating gene expression in single targeted cells needs to be explored. To facilitate the detection of a successful gene activation event whilst developing the technique, the laser will be used to activate the expression of reporter genes such as lacZ and GFP, whose activity can be easily detected. In order to provide a viable method of single cell gene activation, it must be

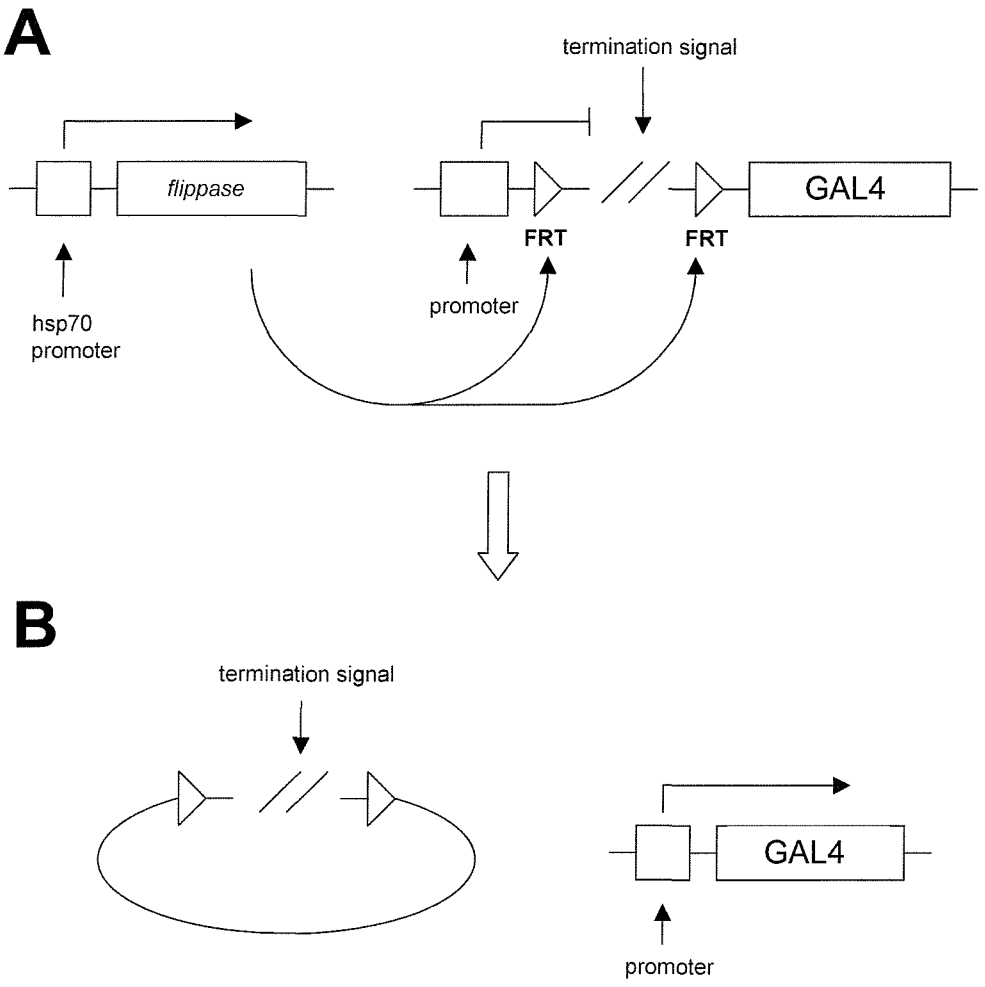


Figure 5.1 The FLP-FRT GAL4 site specific recombination system.

A. The hs-FLP element and the FRT -GAL4 prior to induction of the FLP recombinase. A construct which contains a ubiquitous promoter coupled to the GAL4 gene is interrupted by the insertion of transcriptional termination signals bounded by direct repeats of the FRT sequence. In the absence of FLP activity, transcription is initiated at the ubiquitous promoter but is terminated by these stop signals shortly before the GAL4 coding sequence. **B.** Heat-shock results in the induction of FLP recombinase, which catalyses recombination between the two FRT sites in those cells responding to the stimulus. This leads to the excision of the stop signal that lies between the Actin promoter and the GAL4 coding sequence, enabling GAL4 transcription and expression of genes under GAL4 control.

demonstrated that laser induced heatshock can reliably activate gene expression in targeted cells and produce high levels of constituitive gene expression. An acceptable frequency of laser induced gene expression would be approximately 25%, as this is close to previously described success rates (A. Chiba, personal communication), although ideally a reliability of ≥50% would be achieved.

5.3 Materials and Methods

5.3.1 Fly stocks

All flystocks were maintained on standard Drosophila media (Appendix A) at 18^{0}C. Fly stocks used: hs-lacZ (Bloomington stock centre, USA); hs-GAL4 (Bloomington stock centre, USA); UAS-GFP (Bloomington stock centre, USA); a hs- FLP insert on the third chromosome (Kei Ito, University of Tokyo, Japan); a hs-FLP insert on the first chromosome (Kei Ito, University of Tokyo, Japan); and a heterozygous AyGAL4-UAS-GFP insert on the second chromosome over CyO (hereafter known as FRT-GAL4 UAS-GFP). For all crosses virgin females were collected from the line containing the heatshock promoter fusion and crossed with males from the UAS-reporter gene line.

5.3.2 Conventional heatshocking of embryos

Embryos were collected either overnight or for timed collections of two hours on apple-juice agar plates (Appendix A) with additional yeast. The apple-juice plates were sealed using parafilm and embryos were heatshocked in a water bath at 39^{0}C for 40 minutes.

5.3.3. Preparation of embryos for lasering

Embryos were collected either overnight or for timed collections of two hours on apple juice agar plates (Appendix A) with additional yeast. For initial screening, embryos were manually dechorionated by adhering each embryo to a slide coated with double-sided sellotape and gently popping the embryo out of the chorion using a pair of blunt forceps. Embryos were then transferred to a glue-coated coverslip (double-sided sellotape dissolved in heptane) and covered with 10S Voltalef fluorocarbon oil to prevent desiccation. The coverslip was mounted on a slide using slivers of plasticine to hold the coverslip in place and prevent the embryos from being squashed.

5.3.4 Preparation of larvae for lasering

Larvae were placed on a slide under Drosophila Saline (Appendix A) in groups of 10-20 and pre-screening of larvae for GFP expression was performed using a Zeiss Axiophot FS fitted with epifluorescence under the 10x objective. Larvae of the correct genotype were left to recover for 1-2 hours before being individually treated with the laser. Each larva was

anaesthatised using diethyl ether and placed under a coverslip in Drosophila Saline (Appendix A).

5.3.5 Laser-treatment of embryos and larvae

Cells were visualised using a Zeiss Axiophot FS fitted with Nomarski. Laser heatshocks were performed using a pulsed dye laser (VSL 33: Laser Sciences, Newton, MA). Laser light was delivered through a Micropoint Laser System (Photonics Instruments, Alington Heights, IL) containing the laser dye Coumarin 440. Lasering was performed using either the 63x or the 100x oil objective. A 75-120s laser burst of 4ns pulses at a frequency of 3-5Hz was applied to targeted cells.

5.3.6 X-gal detection of gene expression in embryos

After lasering embryos were returned to apple juice agar plates and allowed to recover under oil at 18^{0}C for 2-4 hours. To examine β-galactosidase expression, embryos were washed off the coverslips with heptane and then transferred to a 1:1 mixture of heptane: 4% paraformaldehyde and fixed for 20 minutes. The fixative layer was replaced by Heptane and the embryos transferred to an egg basket and left to air dry until all traces of Heptane had evaporated. The embryos were then transferred to a 35mm petri dish lined with double-sided sellotape and covered with 4% paraformaldehyde. Those embryos that adhered to the tape could then be nudged out of their vitteline membranes using a stainless steel minutien pin in a pin holder. The embryos were then post-fixed for a further 20 minutes. Three 15 minute washes with PBS-Tw preceded overnight incubation in 8% X-gal staining solution (Appendix A) at room temperature. Graduated washes with 70%, 90% and absolute ethanol were performed before embryos were transferred to 70% glycerol and mounted in 70% glycerol + gelatin.

5.3.7 Fluorescent detection of GFP activation in larvae

After laser treatment larvae were kept individually and allowed to recover on food plates at 18^{0}C or 25^{0}C for at least 48 hours prior to re-examination for GFP activation in targeted cells. For detection of GFP expression in targeted cells each larva was anaesthatised using diethyl ether and placed under a coverslip in Drosophila Saline (Appendix A) and screened for GFP expression using a Zeiss Axiophot FS fitted with epifluorescence.

5.3.8 Sonication of early larvae (0-3 hours after hatching)

Embryos from timed laying periods of 1-2 hours were aged to 22 hours, and those larvae that had hatched were collected and rinsed in bleach for 1 minute to remove any debris and washed in tap water to remove all traces of bleach. Larvae were fixed in 1:1 Heptane: 4% paraformaldehyde and then fixed for an additional 5 minutes in 4% paraformaldehyde containing 0.01% Tween-20. The embryos were washed in Methanol and rehydrated in 70% Methanol/PBS, 50% Methanol/PBS and washed three times in PBT. In order to determine the correct sonication period embryos were sonicated at the lowest power setting for 3 second pulses for various durations and at several different amplification settings. During sonication embryos were kept on ice in between sonication bursts. After sonication embryos were washed 2 x 5 minutes in PBS-Tw. Immunohistochemistry was carried out as described in Section 2.2 and the sensory nervous system was visualised using the Mab22c10 antibody (Developmental Studies Hybridoma bank) at a dilution of 1:250 .

5.3.9 Statistical analysis

Statistical analysis of mortality rates was performed using the Chi-squared test, and statistical analysis of rates of gene activation was performed using Fisher's Exact Test.

5.4 Results

5.4.1 Embryonic laser gene activation

Initial experiments to establish a protocol for single cell laser activated gene expression used lacZ as a reporter gene. β-galactosidase activity can be easily detected by the hydrolysis of the colourless substrate X-gal as cells expressing β-galactosidase turn blue in the presence of X-gal. Two different genotypes of embryos were used. A first reporter gene line contained the lacZ reporter gene directly coupled to the *Drosophila* Hsp70 promoter (hs-lacZ), replicating the previously described transient laser gene activation experiment (Halfon et al., 1997). Additionally a line containing the hs-FLP insert was crossed to a line containing the FRT-GAL4 and UAS lacZ constructs recombined onto the second chromosome to produce hs-FLP x FRT-GAL4-UAS lacZ embryos for constitutive laser gene activation. To ensure that the constructs to be used for laser gene-activation were functional it was first necessary to activate the constructs using conventional heatshock. Embryos at a variety of developmental stages were conventionally heatshocked for both genotypes. This resulted in β-galactosidase activity being detected in approximately 60% of hs-lacZ embryos. Expression was observed throughout the embryo in early stage embryos and in all tissues in later stage embryos (data not shown). To allow sufficient time for reporter gene activation, the hs-FLP x FRT-GAL4 UAS-lacZ embryos were kept at 18^0C for 24 hours before being examined. LacZ expression was detected in approximately 10% of individuals but the majority of embryos had hatched and, as the larval cuticle is impermeable to staining reagents, it could not be determined whether β -galactosidase activity was present in these individuals. All remaining embryos were late stage embryos and expression was detected in all tissues (data not shown).

Once the constructs had been shown to be functional, work began on developing a protocol for embryonic laser gene activation. As embryos had to be manually dechorionated for lasering, it was necessary to cover the embryos with a fine layer of voltalef oil to prevent desiccation. This meant that conventional protocols for the X-gal staining of embryos could not be followed. In the previous laser gene activation experiment the protocol of Vincent and O'Farrell (Vincent and O'Farrell, 1992) had been followed. Several minor adjustments (see materials and methods) were made to the method described to achieve an acceptable success rate for processing small numbers of embryos (data not shown).

A series of laser intensity settings were created by placing a range of neutral density filters, which gradually reduce the intensity of the laser beam, in the path of the laser beam. Using

Differential Interference Contrast (DIC) in combination with neutral density filters (ND) further reduced the intensity of the laser beam. Initially, in order to determine a working range for laser induced heatshock, 3-5 unidentified cells at the anterior of embryos of the hs-lacZ genotype were targeted with the laser. Each embryo was treated at a different laser setting and untreated controls were performed simultaneously. The embryos were allowed to develop for another four hours and then stained for β-galactosidase activity. These initial results from using a laser to activate β-galactosidase expression in hs-lacZ embryos are shown in Figure 5.2. In each case a group of three presumptive epidermal cells in relative proximity at the anterior end of the embryo had been targeted.

In Figures 5.2 A and 5.3 B, two cells in close proximity are expressing β-galactosidase with a third cell staining a short distance away (Figure 5.2 A, arrows, Figure 5.2 B, arrowheads. The other staining visible is the result of non-heatshock dependent background β-galactosidase activity in hs-lacZ embryos, which in slightly older embryos is also visible in cells along the tracheal trunks (Figure 5.2 D). As this background activity occurs close to the region where staining has been observed it was possible that this β-galactosidase activity reflected background activity of the construct, rather than targeted laser gene activation. Early expression in the tracheal trunk cells does not correspond precisely however with the area in which potential activity resulting from laser heat shock is observed. Putative laser activated β-galactosidase expression is also observed in Figure 5.2 C, and the stained cells do not appear to be identical to those proposed to be the result of heat-shock dependent activity in Figure 5.2 A and Figure 5.2 B. The ambiguous nature of the results from these initial experiments left it unclear as to whether successful laser gene activation had occurred. In order to reliably attribute β-galactosidase activity in any cell type to laser driven heat shock it was therefore necessary to target cells of known identity.

As the larval cuticle is impermeable to staining reagents, embryos can only be examined for β -galactosidase expression up to stage 16, prior to cuticle formation. Laser treatments must therefore be performed relatively early in development in order to allow time for sufficient transcription to give a detectable signal before they pass stage 16 of development (approximately 15 hours after egg laying (AEL)). Laser treatments were performed on staged embryos either during germ band extension, at stage 9 or 10, or just after the completion of germ band shortening, at stage 13. Embryonic stage 9 and 10 occur approximately 4.5-5.5 hours AEL, by which time the ventral nerve cord is fairly distinct, enabling presumptive neuroblasts to be targeted. As the differentiation of the embryonic muscles begins in stage 11 and 12 and is completed by the end of stage 13, the developing embryonic musculature was

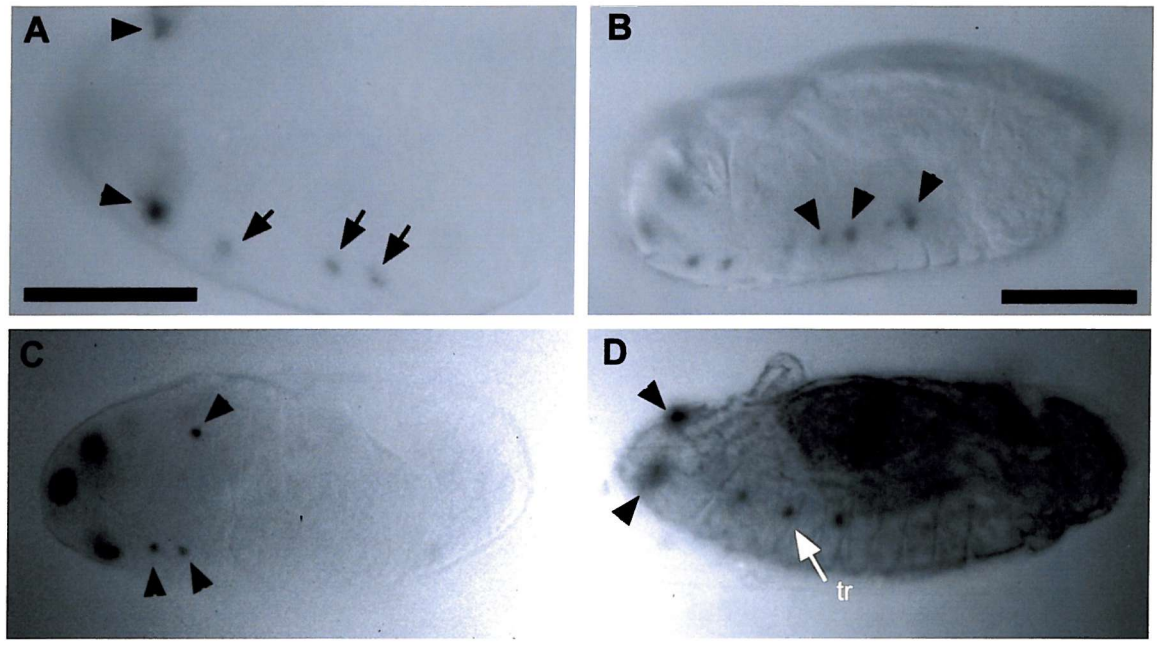


Figure 5.2 Laser-gene activation of β-galactosidase, detected using X-gal. A-C, experimental embryos. D, control embryo. **A.** Expression is observed in the peripheral cells in the anterior region of the embryo close to where cells were targeted (arrows). There is also background expression in a paired anterior structure (arrowhead). **B.** Expression is visible in three peripheral cells close to the region targeted but in a slightly different location from the cells that express lacZ in A. **C.** Staining of a slightly different group of three peripheral cells is shown. **D.** In control embryos, background expression is visible in a paired anterior structure (arrowheads) and in tracheal cells at later embryonic stages (arrow, tr.). (A) Scale bar 0.1mm. (B-D) Scale bar 0.1mm.

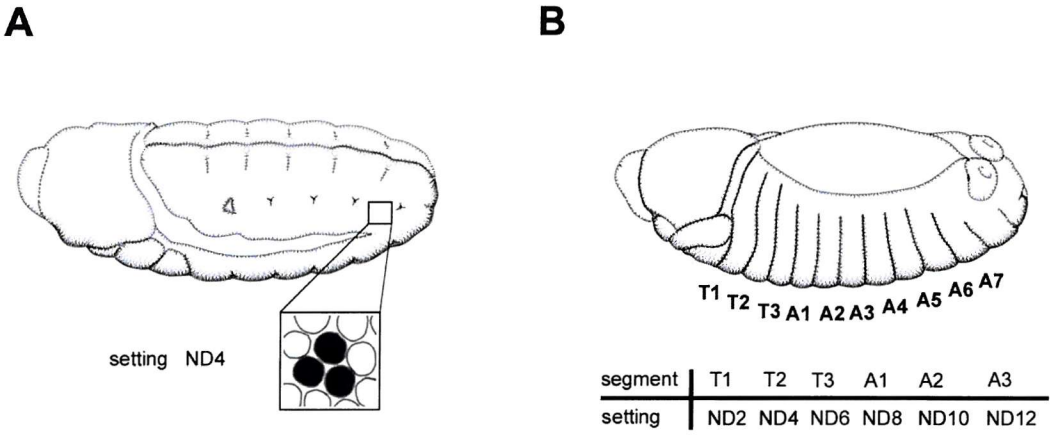


Figure 5.3 Embryonic laser gene activation. A. In stage 9-10 germ band extension embryos, neuroblasts could be visualised. Multiple cells were treated in each embryo at a single laser setting. **B.** In stage 13 embryos, the developing embryonic musculature was targeted. Muscles in each segment could be treated at different laser settings. ND indicates the use of DIC in the settings shown, which further attenuates the laser beam.

targeted in stage 13 embryos (10 hours AEL). In those embryos where neuroblasts were targeted each embryo was treated at a single laser setting. However in those later stage embryos where the developing musculature was targeted and segmentation is clearly visible it was possible to test a different setting in each segment of the embryo. This is shown in Figure 5.3. Graded laser intensities can be tested in each segment from anterior to posterior or alternatively, a single laser intensity can be tested at different durations. The ability to easily test a number of settings within a single embryo coupled with the greater ease of identification of presumptive muscle targets meant that the majority of embryos were targeted as stage 13 embryos. Whether targeting neuroblasts or muscles, in both cases untreated controls were performed simultaneously.

Despite testing a wide range of laser settings and durations in over 150 embryos, no conclusive evidence of successful embryonic laser gene activation was obtained. Embryos where laser treatments were performed at stage 13 (10 hours AEL) and processed for X-gal staining 4 hours later resulted in no, or very faint and ambiguous signals. Embryos of both the hs-lacZ genotype, which would result in transient lacZ production, and the hs-FLP x FRT-GAL4 UAS-lacZ genotype, which would result in constitutive transcription of lacZ, were tested. Activation of lacZ gene expression in neuroblasts at earlier stages in development enabled embryos to be assayed up to 10 hours after being treated, increasing the likelihood of detecting a successful activation event with hs-FLP x FRT-GAL4 UAS-lacZ embryos. However the absence of any independent means of visualising cells meant that it was difficult to be sure that the intended cell(s) had been targeted.

To reliably determine whether laser activated gene expression is being successfully achieved the cellular target needs to be able to be accurately identified. Accurate identification of individual cells is extremely difficult in early embryos, where tissues are relatively undifferentiated but laser treatments cannot be performed later in embryonic development once all embryonic structures are fully differentiated unless some means is found of permeablising the larval cuticle to staining reagents, otherwise reporter gene activation could not be detected. Sonication of late stage embryos (Stage 17+) and larvae, to permeablise the cuticle to certain antibodies and staining reagents, has been described (Patel, 1994) and could provide a means of detecting reporter gene activity if laser treatments were performed on fully differentiated embryos. Although sonication enables staining reagents to pass through the larval cuticle it can also cause significant morphological damage (Patel, 1994). Sonication as a means of visualising reporter gene activity at later development stages was investigated using the Mab22c10 antibody which labels the sensory nervous system, as this antibody produces

extremely reliable staining and has been used successfully with sonication previously (Patel, 1994). First instar larvae (0-3 hours after hatching) were sonicated at a range of settings and durations to try and identify a suitable setting and sonication duration that will result in good antibody staining with minimal morphological damage. The results are shown in Table 5.1.

Table 5.1 Sonication of first instar larvae (0-3 hours after hatching).

Sonication settings	Duration (s)	Effect on larvae
Low; Ampl. 4	3	Poor staining, little morphological damage
Low; Ampl. 4	6	Few moderately stained larvae, a small proportion of larvae show morphological damage
Low; Ampl. 4	9	Larvae are either stained but morphologically damaged or show minimal signs of staining.
Low; Ampl. 4	12	Majority of larvae show morphological damage but are well stained
Low; Ampl. 2	9	Good staining in several individuals. Many individuals are well stained but morphologically damaged but a significant proportion show minimal or no staining.
Low; Ampl. 2	12	Larvae are either stained but damaged or show minimal signs of staining.

All groups contained a reasonably high proportion of larvae that show little or no staining. These larvae tend to be slightly larger than their stained counterparts. This slight difference in size may determine the susceptibility of the larvae to sonication and these larvae may therefore require a longer sonication period that would destroy the smaller individuals. This difference in size may reflect slight differences in age or may purely reflect differing physical strength and metabolic rates between individuals. For all settings tested, almost all larvae that were well stained also showed signs of morphological damage. It was therefore decided not to pursue sonication as a means of permeablising the larval cuticle to enable cells to be targeted later in embryonic development.

As an alternative to embryonic laser gene activation cells could also be targeted in late stage larvae. There are a number of advantages to targeting cells in larvae rather than embryos. Firstly, cellular targets such as individual sensory neurons can be easily identified in larvae,

which will enable successful laser gene activation events to be reliably detected. Secondly, late stage larvae are sufficiently large to be dissected, bypassing the impermeability of the larval cuticle to staining reagents, enabling reporter gene expression to be visualised. Thirdly, the technical difficulties related to processing very small numbers of embryos are removed. By targeting cells in larvae a protocol for single cell laser gene activation can be established, which can then be tested in the embryo. All further efforts were therefore concentrated on laser gene activation in larvae.

As GFP reporter gene constructs were becoming more widely available I also decided to use GFP as an alternative reporter gene to β-galactosidase to further aid detection of reporter gene expression. As GFP can be visualised directly in live material using epifluorescence, laser-treated larvae can be quickly screened to determine whether GFP expression has been successfully activated. In addition, each larvae can be screened at a number of different time points after the laser treatment to determine when GFP expression becomes apparent in the targeted cells.

5.4.2 Laser gene activation in larvae

5.4.2.1 Transient activation of GFP in targeted cells

A transgenic line containing the hs-GAL4 promoter fusion construct was crossed to a line containing the UAS-GFP reporter gene. Progeny were conventionally heatshocked as embryos to ensure the construct was functional (data not shown). All laser treatments were performed on late 2nd or early 3rd instar larvae. Again, successive neutral density filters, with or without DIC, were used to create a series of laser settings that were tested individually on single larvae at laser treatments of between 75 and 120 seconds. Those settings that resulted in visible cell damage were excluded and subsequently only lower laser intensity settings were tested. Sensory neurons can be easily identified in third instar larvae and, to increase the chances of successful activation, multiple cells were subjected to the chosen laser treatment in each individual. The ventral cluster of sensory neurons which contains five multidendritic neurons, including the bipolar dendritic neuron vbd, was selected as a target and four or five ventral cluster cells were targeted with the laser in each experimental individual and sham-treated in controls.

Larvae were initially examined at various time points after lasering but repeated examination resulted in almost 100% mortality by the later time points (data not shown), so subsequently

larvae were examined either at two, three, four, twelve or twenty-four hours after lasering. The survival rates resulting from each laser setting is shown in Figure 5.4. This shows that although there is a general trend of increased mortality as the power of the laser beam increases, both with and without DIC, there is no statistically significant difference in mortality between treated and untreated control individuals for any laser setting. This indicates that laser treatment does not significantly influence larval mortality.

Out of 107 treated individuals, successful laser activation was achieved in only one larvae, although all five target ventral cluster cells showed expression (Figure 5.5). The resulting expression was at a very low level however, insufficient to label the entire axonal trajectory. If gene activation using the hs-GAL4 system resulted in such a faint signal, previous successful activation events could have been missed. It was already difficult to determine whether or not gene activation had occurred because such expression would be transient. The absence of any observable expression could therefore either indicate that expression has not been activated or that the larvae are being assayed either too early or too late for GFP to be detected. This problem is further compounded by the low level expression resulting from laser-induced heat shock, as it is likely that the signal would only be detectable for a very short period.

Although successful laser activation of GFP expression was only observed in one individual, this served to demonstrate that our experimental set-up was capable of activating gene expression by laser-induced heatshock. The difficulties associated with visualising transient GFP expression meant that once a successful reporter gene activation event had been observed all further efforts to develop a means of laser induced gene expression in single cells were concentrated on the modified version of the laser gene activation technique described in the introduction. This would enable reporter gene expression to be constituitively activated, thus removing the problems of transient expression for the detection of successful laser gene activation events and for determining reliability. Permanent activation of gene expression would also mean that GFP would be continually being driven at a high level in targeted cells. The entire neuronal morphology of the target cell should therefore be visible, meaning that the axon of the targeted cell should be able to be traced into the CNS.

5.4.2.2. Constituitive activation of gene expression in targeted cells

A transgenic line containing the hs-FLP element was crossed to a line containing both the FRT-GAL4 construct and the UAS-GFP reporter gene. Progeny were first examined for background expression (n=10), which was apparent in a stereotyped cluster of cells

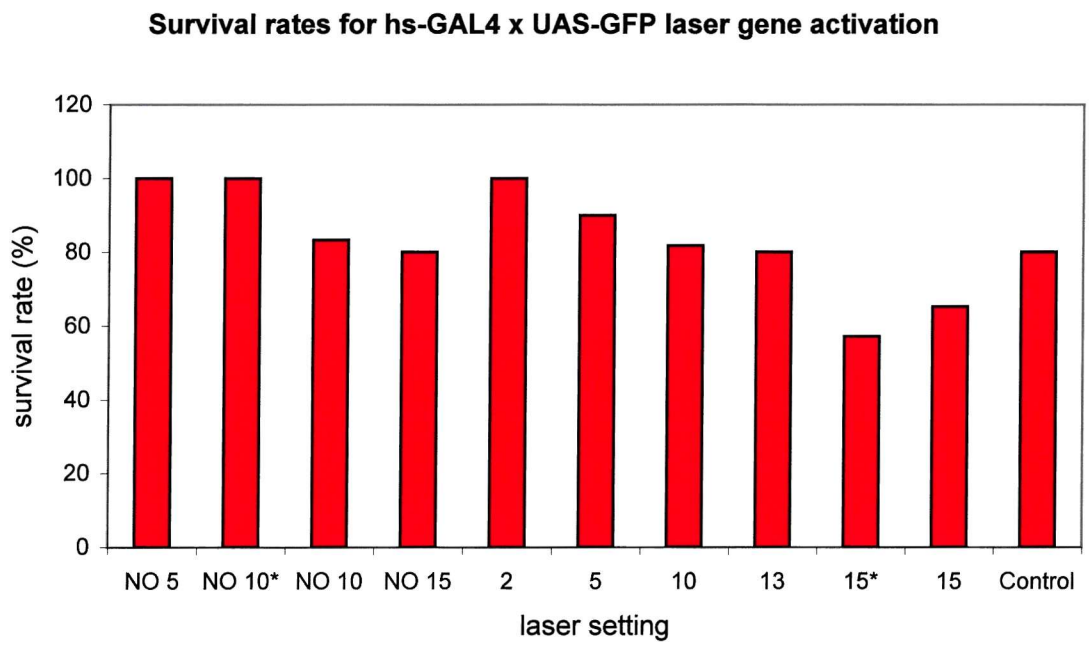


Figure 5.4 Survival rates after transient laser gene activation. Number indicates a decrease in neutral density filters, such that 5 is the weakest setting and 15 is the strongest setting. NO indicates the use of DIC to further attenuate the laser beam. Statistical analysis using the Chi-squared test shows that the differences between the survival rates for the different laser treatments are not significant.

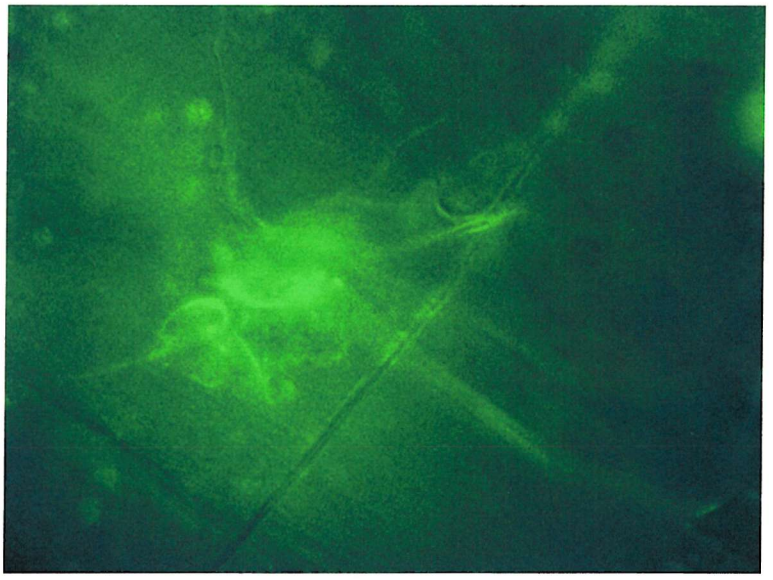


Figure 5.5 Transient laser gene activation. Shows the successful activation of GFP expression in all target neurons. GFP is expressed at a low level in all ventral cluster neurons.

surrounding the anterior and posterior spiracles and also in a subset of CNS cells. However, the absence of any visible expression in the periphery in abdominal segments made this line suitable for establishing a constituitive laser gene activation protocol in sensory neurons. Laser treatments were performed on either 2nd or early 3rd instar larvae, which were assayed as late 3rd instar larvae to ensure that any GFP present should be detectable. Again, successive neutral density filters, with and without DIC, were used to create a stepwise range of laser setting at which larvae could be tested using laser treatments of between 75 and 120 seconds.

Initially larvae were laser-treated blind to genotype but as the line containing the FRT-GAL4 and UAS-GFP constructs (which are recombined onto a single chromosome) is heterozygous, only 50% of treated individuals were of the correct genotype. Due to the significant time and effort required to generate treated material, it was subsequently decided to pre-screen larvae prior to laser treatments to ensure that they carried the FRT-GAL4 and UAS-GFP constructs. This could be determined by the presence of background GFP expression surrounding the posterior spiracles, which would only be apparent in those larvae containing the FRT-GAL4 and UAS-GFP constructs. Larvae were therefore pre-screened in batches of 20 using epifluorescence at 10x magnification. Larvae of the correct genotype were left to recover for 1-2 hours before being individually treated at a specific neutral density setting, with or without DIC, and then allowed either 48 hours or 36 hours to recover before being examined. Controls were generated using both untreated larvae and larvae that had undergone sham laser-treatments.

Initially two different hs-FLP constructs were tested simultaneously but once GFP activation was seen with one line, all further experiments concentrated on that hs-FLP line. A number of individuals showed GFP expression in epidermal cells overlying or surrounding the targeted cells at several settings and several settings were found which resulted in reporter gene expression in targeted neurons. A successfully targeted neuron is shown in Figure 5.6. High levels of GFP resulted, meaning the axon and dendrites of the targeted cell could be easily visualised and increasing the likelihood that the axonal projections within the CNS would be clearly revealed. Chance observation of untargeted segments however also revealed GFP expression in non-targeted sensory neurons. While larvae had been quickly pre-screened prior to lasering, none had been examined for sensory neuron expression prior to lasering as no sensory nervous system expression had been observed when untreated progeny had been examined for background expression (n=10). It was therefore important to determine whether such expression was the result of the laser treatment or whether it was already present prior to the laser treatment. Additionally, if random events occur at a high frequency, perhaps the

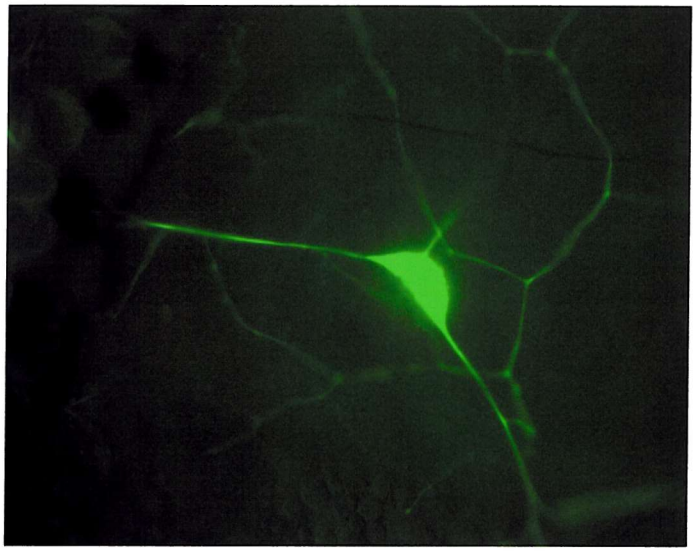


Figure 5.6 Constituitive laser gene activation. Shows the successful activation of gene expression in a target ventral cluster md-da neurons. GFP is expressed constituitively and at a high level throughout the neuron, enabling the axons and dendrites to be visualised.

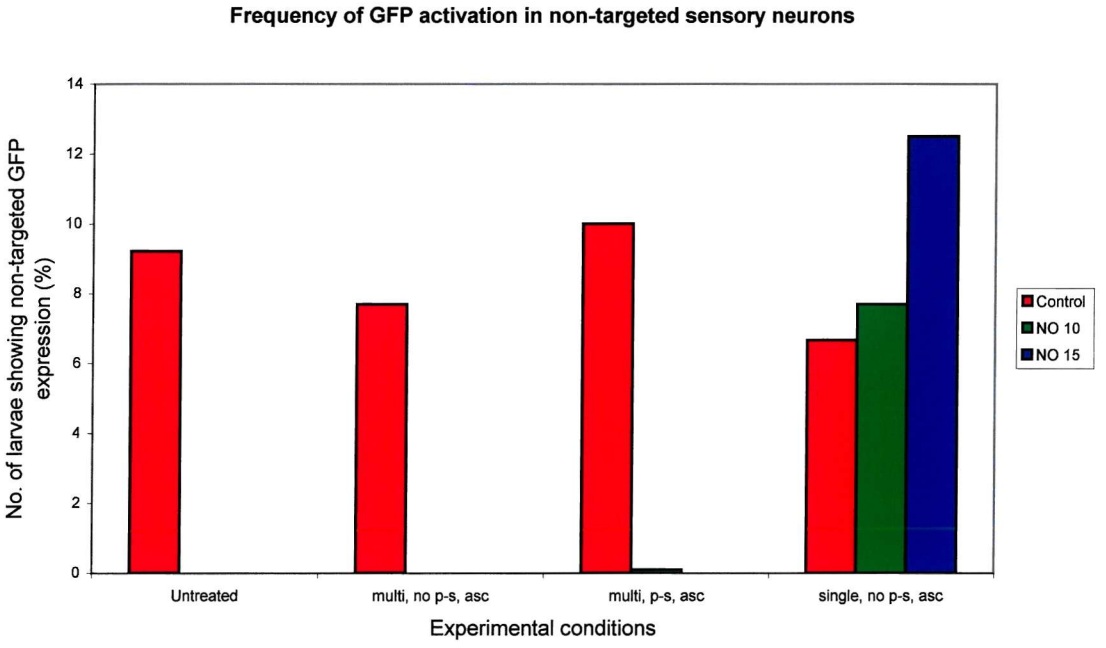


Figure 5.7 Frequency of GFP activation in non-targeted sensory neurons. multi - multiple cells targeted, single, single cells targeted, p-s - pre-screening, asc- all segments checked prior to lasering, snc - segments not checked prior to lasering. In all cases $n \geq 10$ and for the untreated larvae n=141. Statistical analysis using the Chi-squared test shows that differences in GFP activation between different laser treatments are not significant.

expression observed in targeted neurons reflects a chance event rather than successful laser gene activation.

In order to test this possibility, the ventro-lateral region of each abdominal segment in A1-A7 was screened prior to lasering and those showing GFP expression were discarded. The same region was then re-examined in A1-A7 after lasering to determine whether GFP had been activated in any of the sensory neurons within this area. As heat shock promoters respond to a variety of different environmental stresses, not just heat, these random FLP activation events may have been a consequence of stress to the animal as a result of the lasering procedure. As prolonged exposure to UV light might be a contributing factor, the effects of pre-screening on the background rate of FLP activation was examined by comparing FLP activation rates between pre-screened and non-pre-screened larvae. Additionally, in order to minimise the length of the procedure a single cell was targeted in each individual, as opposed to the previous targeting of multiple cells in each segment and, occasionally, in multiple segments. By generating sham-treated controls in each case the effects of manipulating such variables on the background rate of FLP activity could be determined. These are summarised in Figure 5.7.

These experiments revealed a background rate of GFP activation in non-targeted cells of approximately 10 percent in untreated larvae, which is not significantly affected by pre-screening or by the duration of the laser procedure. The slight variations between treatments were not statistically significant. The rate of GFP activation in non-targeted cells was also recorded for each laser protocol (only those treatments for which there is an n of greater than 10 are shown in Figure 5.7). Different sham treatments again did not significantly affect the rate of GFP activation in non-targeted cells and background rates of GFP activation are therefore not a consequence of the laser protocol per se (Figure 5.7).

It is therefore unlikely that the GFP expression observed in targeted cells reflects background random GFP activation and may therefore result from laser-induced heatshock in the targeted cells. A comparison of the rates of GFP activation in target cells between controls and laser treated larvae is given in Figure 5.8. This figure summarises the percentage of times a laser treatment resulted in GFP activation and shows that GFP activation in targeted larvae occurs at a rate of 37.5 percent, and with random FLP activation in targeted cells occurring at a frequency of 10 percent, this gives a laser activation rate of approximately 25 percent. However, while five cells were targeted in each larvae, GFP expression was only ever observed in one targeted cell out of five, giving a true value for successful laser gene activation of 10 percent, with GFP expression being observed in targeted cells in sham-treated larvae at a

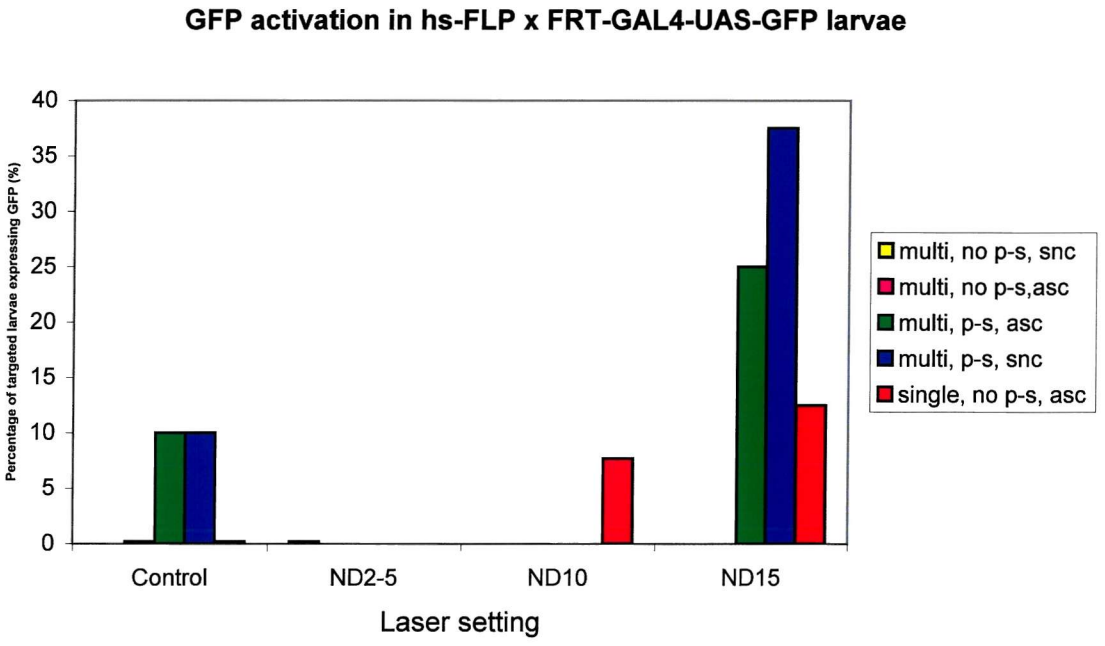
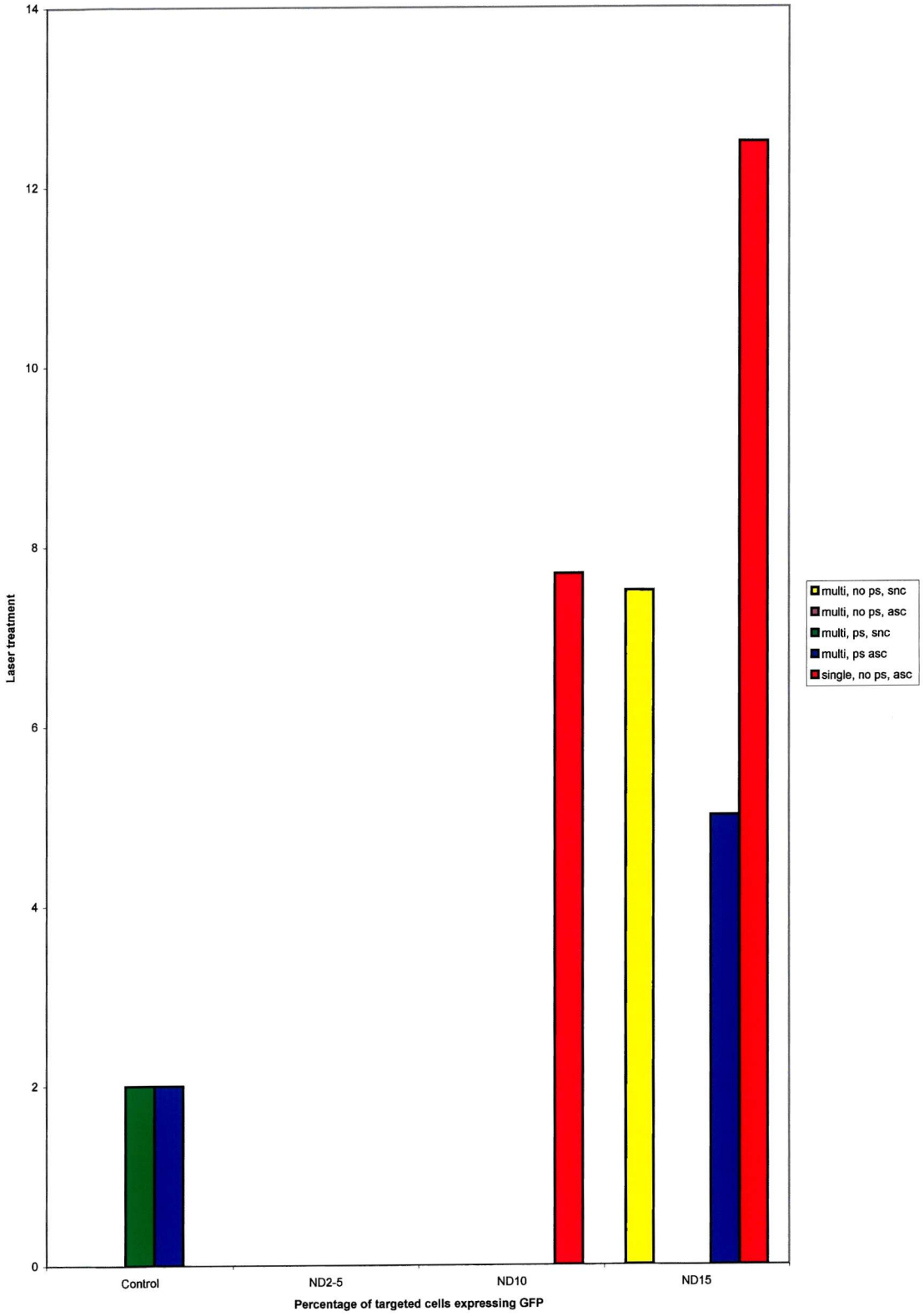


Figure 5.8 Constituitive GFP activation in targeted larvae. multi - multiple cells targeted, single- single cells targeted, p-s - pre-screening, asc- all segments checked prior to laser treatment, snc - segments not checked prior to laser treatments. Statistical analysis was performed using Fisher's Exact Test and showed that the activation of GFP expression in targeted larvae compared to controls is significant ($p<5\%$).

Figure 5.9 Constituitive activation of GFP expression in targeted cells. multi - multiple cells targeted, single- single cells targeted, p-s - pre-screening, asc- all segments checked prior to laser treatment, snc - segments not checked prior to laser treatments. Statistical analysis was performed using Fisher's Exact Test and showed that the activation of GFP expression in targeted cells in laser treated larvae was not statistically significant.

Frequency of GFP activation in targeted cells



frequency of 2 percent (Figure 5.9). The rate of gene activation in target cells as a result of laser induced heatshock is therefore so low as to make laser induced gene activation impractical as a means of targeting gene expression to single cells.

The effect of each setting and lasering conditions on larval survival rates was also analysed and is summarised in Figure 5.10. This showed that the survival rates from most laser treatments did not differ significantly from the survival rates for sham treated controls although the increase in mortality resulting from the highest intensity laser treatment was statistically significant ($p<0.05$). As treatments of higher intensity did not produce a statistically significant increase in mortality in hs-GAL4 x UAS-GFP larvae, this is not likely to truly represent a deleterious effect of laser treatments on larval viability at the intensities used during these experiments. As n numbers for all groups are small, the difference could largely reflect chance variations in viability on those days due to external factors.

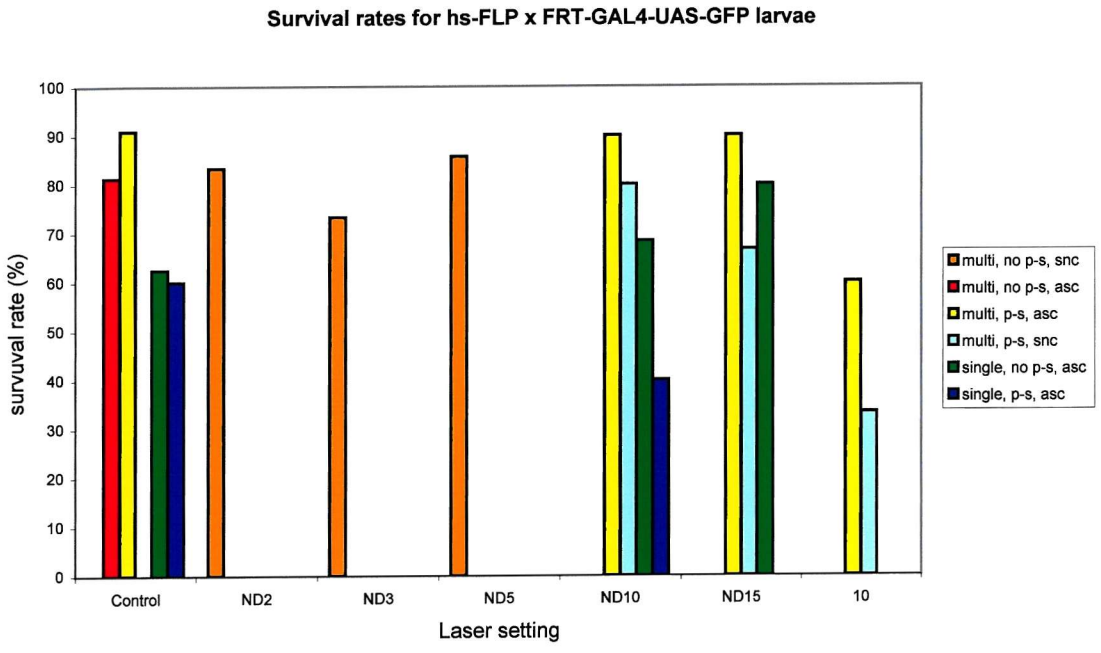


Figure 5.10 Survival rates for constitutive laser gene activation. multi - multiple cells targeted, single- single cells targeted, p-s - pre-screening, asc- all segments checked prior to lasering, snc - segments not checked prior to lasering. Statistical analysis using the Chi-sqaured test shows that the differences in survival rate between different laser treatments are not significant, except for the highest intensity laser treatment ($p<5\%$).

5.5 Discussion

The aim of this work was to examine the potential of laser-activated gene expression as a means of targeting gene expression to single cells. Although laser induced gene expression would need to be activated in the embryo if the effects of candidate gene misexpression on the specification of central projection pattern were to be addressed, due to the difficulties of reliably establishing in the embryo whether reporter gene expression was been activated as a result laser induced heatshock, it eventually proved more practical to develop the technique in larvae and then transfer the technology back to the embryo. The observation of successful activation of reporter gene expression after transient gene activation indicated that the laser was capable of activating gene expression in targeted cells, but in order to reliably determine the frequency with which gene expression was being activated the constituitive activation of gene expression was required. Although constituitive activation of gene expression was eventually successfully achieved, random activation of gene expression in non-targeted cells also occurred and laser induced gene expression occurred at such a low rate as to be impractical when compared to the amount of effort required to generate laser treated material.

5.5.1 Arbitrary nature of laser settings

The exact power of the laser beam can fluctuate significantly and can only be approximately estimated by determining the lowest setting at which the laser can crack a coverslip, a procedure performed before every lasering session to provide a rough calibration of the laser's intensity. While the laser in general shows little change in intensity over a period of several weeks, it can also change dramatically over a period of a few hours. This means that, despite calibration at the beginning of a session, the power ascribed to each laser setting is not necessarily constant. Indeed the successful activation event observed using hs-GAL4 UAS-GFP larvae was recorded on a day where at the end of the session the laser was discovered to have attenuated significantly. The intensity of the laser also decayed gradually over time. Replacement of the dye cell, which occurred during the course of these experiments, dramatically increased the power of the laser. Each individual laser setting is therefore somewhat arbitrary and can only be considered to be an approximate value.

5.5.2 Difficulties associated with embryonic laser gene activation

Attempts at achieving successful laser-induced embryonic gene activation were primarily hampered by the difficulties associated with examining laser-treated individuals as embryos.

Firstly, the use of the hs-lacZ construct has the associated problem that any resulting activity will be transient. The absence of any observable expression could reflect the fact that embryos are being assayed either too early or too late for β-galactosidase expression to be detected, rather than that no successful gene activation has ever occurred. Conversely, while the use of the hs-FLP FRT-lacZ construct results in constitutive expression of the lacZ gene in targeted cells, a significant amount of time may have to elapse before such expression becomes detectable. Such expression may not even be observed within the timeframe of embryonic development. This construct was therefore primarily targeted at stage 9-10, when it could be examined approximately 10 hours later. The necessity of targeting embryos at a sufficiently early stage in embryonic development for enough time to elapse to produce a detectable signal but to still be able to be examined as embryos produces a further problem. Clearly differentiated embryonic structures do not appear until the late stages of embryonic development - sensory neurons for example cannot be reliably identified until embryonic stage 15-16. This means that confident identification of cellular targets at earlier stages of development is difficult and requires experience. It is therefore hard to be sure that the intended cells have been targeted.

Additionally, embryos can only be laser-treated in small groups and were generally treated in groups of 10, consisting of 5 experimental embryos and 5 control embryos, which would have to be processed separately. This presents a technical problem in the processing of very small number of embryos, all of which require hand-devittelinisation. A number of embryos were therefore lost during processing, particularly while the technique was being perfected.

5.5.3 The low level of transcription resulting from hs-GAL4 activation

An extremely low level of GFP expression was observed to result from laser-induced hs-GAL4 activation. Such low activity may reflect the fact that a significant number of GFP molecules, possibly as many as 50,000 per cell (C. O'Kane, personal communication), need to be produced in order to result in a detectable signal. Due to the short length of the stimulus, GAL4 will only be produced for a short time in each cell in which the heat-shock response is activated and the amount of GAL4 produced may not be sufficient to drive a high level of gene expression from its target promoter, the UAS response element.

Coupled with the transient nature of this expression and the resultant difficulty in accurately assaying activity previously discussed, successful activation events could easily go undetected. Such difficulties are not unique to this project however and hs-GAL4 elements have been

reported elsewhere to result in low levels of target gene expression (A. Chiba, personal communication).

5.5.4 Why did non-specific activation of GFP occur in non-targeted cells?

The GFP expression observed in non-targeted cells could still reflect appropriate activation of the Drosophila heatshock promoter. Heatshock proteins, originally identified on the basis of their induction upon heat shock, have since been found to be induced in response to a wide variety of different types of cellular stress and might therefore be more accurately described as stress-response proteins. There are a number of different potential sources of cellular stress during the laser procedure that could cause the activation of the heat-shock promoter in non-targeted cells. These include the etherisation procedure required to anaesthetise the larvae, the pressure of the coverslip over the larvae, the exposure to UV light during the screening process, and the lasering itself which could result in non-specific heating of surrounding cells. Despite these various sources of cellular stress, the rate of random GFP activation is no greater among untreated larvae than those that have been subjected to various sham-treatments. The observation of a similar frequency of GFP expression observed in non-treated larvae may therefore reflect the occurrence of a stress-related event during rearing, perhaps a consequence of crowded conditions in some particular batches or of larval competition for food resources.

An alternative and perhaps more likely explanation is that there is mosaic background activity associated with either the hs-FLP insert or the FRT-GAL4 UAS-GFP insert. Background expression in the hs-FLP line cannot be assayed independently of a reporter gene line but by comparing the levels of background expression seen with different hs-FLP inserts, if such activity is seen with one line and not another, then the background activity must be caused by mosaicism within the hs-FLP line. Background expression is observed in the FRT-GAL4 UAS-GFP line in the absence of the hs-FLP construct however, suggesting that the transcriptional repression of the GAL4 transgene is incomplete and that there is leaky GAL4 activity. Such activity was observed in the same structures where background expression was observed in the progeny of the hs-FLP x FRT-GAL4 UAS-GFP cross. It is therefore likely that the low frequency of background GFP observed in non-targeted cells is a result of mosaicism associated with the FRT-GAL4 insert. As discussed in Chapter Three, mosaic gene silencing can be caused by position effect variegation, which can occur when a transgene inserts close to a heterochromatic region of the genome. A significant level of background activity in the FRT-GAL4-UAS-GFP line could therefore be further subject to position effect variegation, resulting in occasional expression in sensory neurons.

5.5.5 Why were random activation events only seen with the FLP construct and not with the GAL4 construct?

There are two main explanations as to why random activation events should be seen with one genotype and not another in the same experimental situation. Firstly, as discussed above, epigenetic factors could influence gene expression and the different locations of the site of P-element insertion in the different transgenic lines could cause the differences in transgene expression and background activity. Alternatively, the level of transcriptional activation required to produce a functional level of FLP recombinase may be far lower than the level of transcriptional activation required to produce a functional level of GAL4 transcription factor. It is theoretically possible that just four molecules of FLP would be required to catalyse recombination, as they bind tightly and specifically to the FRT site (M. Boocock, personal communication). One dimer of GAL4 is required to drive transcription from the UAS promoter therefore, as there are 5 repeats of the UAS-promoter, a minimum of 10 molecules of GAL4 would be required to achieve sufficient GFP expression. It is also likely that GAL4 would also bind to many other similar sites around the genome and would also bind non-specifically to DNA in general, meaning that a realistic number of GAL4 molecules required for detachable GFP expression would probably be hundreds or thousands per nucleus - several degrees of magnitudes greater than that required for FLP at any rate. The activation thresholds for the two different methodologies may therefore be significantly different.

5.5.6 Consequences of random activation events for targeted gene expression

The realisation that GFP was being activated in non-targeted cells was a huge disappointment after investing a large amount of time in getting the technique up and running. Concurrently, a new technique of tissue-specific mosaic analysis was published which provided a means of examining not only gain-of-function but also loss-of-function mutations in single neurons. This seemed a significant advantage over the laser induced gene expression, which could only be applied to ectopic expression phenotypes with the constructs available. Coupled with the lack of success in reducing random activation events and the low rate of successful target gene activation, I decided to abandon the laser-activated gene expression project and concentrate my remaining time on tissue specific mosaic analysis.

On reflection however, random activation may not in fact be such a large problem as it appeared at the time. Random events are only truly a problem if they occur in the same

segment as targeted gene expression, as they could then interfere with the analysis of the central projection pattern of the targeted cell. Random activation in other segments however would have little or no consequence for the examination of the targeted sensory neurons. In addition the unique advantage of laser-activated gene expression is the ability to choose the target cell.

5.5.7 Visualisation of target cells

An enduring problem is that, without any independent means of visualising the cells to be targeted, it is impossible to be absolutely certain that the correct cell has been targeted. While the cells of the ventral cluster can be easily visualised by eye, it is unclear whether the nuclei of a given sensory neuron is in the same plane as the focus of the laser beam. Indeed, it often appears that the plane in which a cell lies when visualised using light microscopy is slightly different from the plane in which that cell lies when visualised using fluorescence. Perhaps when a sensory neuron is targeted using light microscopy the actual cellular target overlies the supposed sensory target. Significantly, GFP expression is often observed in overlying epidermal cells although whether this reflects successful activation of gene expression but inaccurate targeting is unclear. Activation of GFP in surrounding cells could also result if the heat produced by the laser is so great that it prevents the heatshock response, which is inhibited at temperatures above 40^0C, from occurring but that sufficient heat spreads to neighbouring cells to activate the heat shock response in them.

5.5.8 Future improvements to the technique

The recent isolation of novel red fluorescent proteins (RFPs) from the Indo-Pacific coral *Discosoma,* which have absorption and emission spectra similar to rhodamine, provides a means of addressing the problem of visualisation of target cells. A second reporter could be created that was independent of the GAL4-UAS system by fusing the pan-neuronal elav promoter to the coding sequence of the RFP, providing a means of independently visualising target cells.

An alternative approach would be to identify a GAL4 line that is expressed in sense organ precursors but has no expression in the mature PNS or CNS, thereby enabling target cells to be visualised early in their development but not affecting the visualisation of targeted cells later in development. While such an approach also makes use of the GAL4 system, it should not interfere with targeted GFP reporter gene activation as the more global pattern of UAS-GFP

expression driven by the GAL4 line will be switched off early in development, leaving only those cells in which GFP has been constituitively activated through laser-induced heatshock visible. This could be extremely useful for analysing the fate of a specific cell throughout development. Such an approach might have significant drawbacks for targeted gene misexpression however, as all such genes would also be misexpressed in the sense organ precursors labeled by the early phase of GAL4 expression, and could therefore severely disrupt neuronal development.

Chapter 6. MARCM (Mosaic Analysis with a Repressible Cell Marker)

6.1 Aim

The potential of the MARCM technique as a means of visualising sensory axons in the CNS was investigated, with the aim of characterising the normal pattern of central projections for each embryonic sensory neuron in the larval CNS. This analysis will provide a framework in which the MARCM technique can be used to examine the function of candidate genes in specifying axonal morphology.

6.2 Introduction

Mosaic analysis has a long history in *Drosophila* and has enabled pioneering ideas, such as the notion of compartments, to be tested; ideas which have become part of the foundation of modern developmental biology. By creating clones of genetically distinct cells within an organism so that the organism becomes "mosaic" for a particular mutation it has been possible to examine the role of particular genes within specific tissues, and to explore fundamental biological questions such as autonomy of gene action and restriction of cell fate (Perrimon, 1998).

Mosaics are generated by inducing mitotic recombination between homologous chromosomes in heterozygous individuals. Traditionally, X-rays have been used to cause chromosomal breaks, which can result in exchange between homologous chromosome arms. Daughter cells homozygous for a given mutation or marker gene can be formed after mitosis, creating a somatic clone of mutant cells. Mitotic recombination induced by ionising radiation occurs at a low frequency however, and achieving an acceptable frequency of clones often causes significant cell death and can result in developmental abnormalities unrelated to mosaicism (Xu and Harrison, 1994).

To create a more efficient method of generating mosaics the FLP-FRT site specific recombination system of the yeast 2μ plasmid has been adapted for use in *Drosophila* (Figure 6.1). Initial studies placed the FLP recombinase gene under the control of the hsp70 promoter and used the *white* gene, which is essential for the production of eye pigment, as a marker (Golic and Lindquist, 1989). Heat-shock results in the induction of FLP recombinase, which catalyses recombination between the two FRT sites in those cells responding to the stimulus. This leads to the excision of the FRT-flanked *white* gene, enabling clones within the

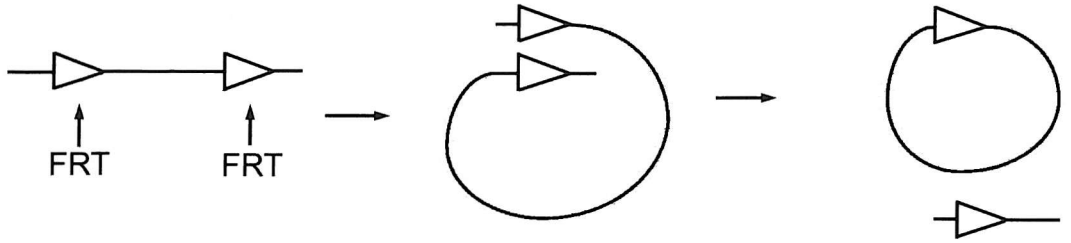


Figure 6.1 Site specific recombination. The basic principle of site-specific recombination between two direct repeats is illustrated. FLP recombinase catalyses recombination between two FLP recombination target (FRT) sites, excising the intervening DNA.

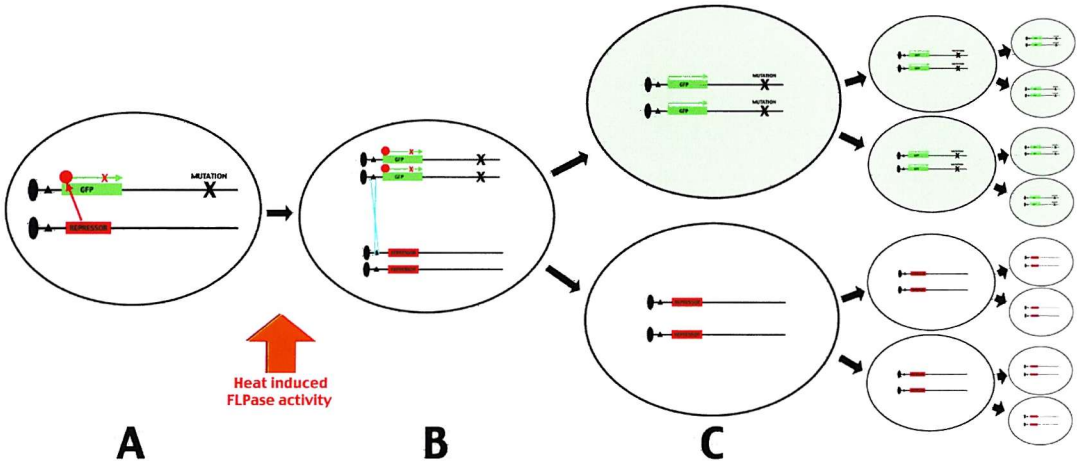


Figure 6.2 The MARCM technique. A. In the absence of heatshock all cells express GAL80, a dominant repressor of GAL4 activity and the UAS-GFP reporter gene is not expressed. **B.** Heatshock induced activation of the FLP recombinase in mitotic cells catalyses recombination between the two FRT sites. **C.** This can lead to the production of homozygous clones of cells that have lost the GAL80 repressor. As the GFP reporter gene is under GAL4 control, GFP expression will only occur if clones are induced in cells in which GAL4 is expressed. This results in the production of positively labelled, tissue specific mosaics.

eye to be recognised by the absence of the marker gene. The system can also be used to induce high frequency mitotic recombination between recombinase target sequences located on homologous chromosome arms (Golic, 1991). By placing a FRT sequence near the base of each major chromosome arm, mosaic animals can be efficiently generated and screened for new mutations affecting many biological processes, including lethal mutations affecting the development and function of adult structures that would not be identified in most genetic screens (Xu and Rubin, 1993).

It is often desirable to be able to positively, rather than negatively, label mosaic cells and FLP-FRT site-specific recombination can also be used to positively identify mosaic clones. A modification of the technique enables genes to be switched on only in those cells where recombination has taken place. FLP-mediated site specific recombination catalyses the excision of transcriptional termination signals that lie between the promoter and the gene to be expressed, removing the block to gene transcription and resulting in the initiation of gene expression (Struhl and Basler, 1993). This creates positively labelled clones of cells expressing a marker gene and can be used to determine cell lineages. Situations in which this technique has been employed effectively include the examination of clonally related cells within complex brain structures such as the mushroom bodies (Ito et al., 1997), and to ascertain the origin of multiple dendritic neurons within the PNS and their relationship to other types of sense organs (Brewster and Bodmer, 1995). The incorporation of the GAL4 enhancer trap technique (See Chapter 3), by including GAL4 as the gene whose expression is switched on by FLP-mediated site-specific recombination, further increases the utility of this approach (Ito et al., 1997).

The major drawback to mosaic analysis, however, is the inability to control in which cells recombination events will occur. This becomes an especial issue when one wishes to examine one specific tissue type and the mutations under investigation occur in patterning genes. For the nervous system in particular it is essential that one can be certain that any defects observed in nervous system formation does not merely reflect an earlier disruption in the patch of epidermal cells from which that part of the nervous system was derived. Embryos homozygous for loss of function mutations in patterning genes therefore cannot be used to examine the effects of these genes in nervous system development. While many such embryos display clear defects in peripheral nervous system organisation, patterning in the epidermis is so disrupted that the PNS defects cannot be dissociated from their developmental history (Patel et al., 1989).

Lee and Luo (Lee and Luo, 1999) have now developed an elegant technique that overcomes these limitations to mosaic analysis (Figure 6.2). MARCM, Mosaic Analysis with a Repressible Cell Marker, uses the GAL4 system to restrict potential mosaicism to those cells where GAL4 is expressed but also includes GAL80, a transcriptional repressor of GAL4, so that in the normal situation there is no GAL4 activity. Again, the GAL4 methodology is combined with FLP-FRT site specific recombination but in this case induction of the FLP recombinase removes GAL80 repression and enables GAL4, and those genes under GAL4 transcriptional control, to be expressed. Cells mosaic for a gene of interest are positively marked by introducing a visible maker, such as Green Fluorescent Protein (GFP), which is also under GAL4 control. The system can also be used to make cells homozygous for a recessive mutation in a gene of interest and again, those cells where recombination has occurred are visualised using GFP.

In order to create mosaicism within the nervous system the elav-GAL4 driver, which is expressed in all cells of the nervous system, can be used. As the FLP recombinase is under the control of a heat shock promoter, by modulating the heat shock conditions used to induce recombination (and consequently removal of GAL80 repression), it is possible to recover single mosaic neurons which can easily be identified through their expression of GFP. This technique therefore provides a means of targeting gene expression to single identified sensory neurons. While laser-activated gene expression also enables genes to be activated in individual neurons, the advantage of the MARCM technique is that it enables both loss of function and gain of function analysis of genes of interest within single sensory neurons.

In addition to targeting gene expression to single neurons it is also necessary to be able to visualise the central projections of those neurons in which genes are being misexpressed. As previously mentioned, the MARCM technique enables mosaic neurons to be positively labelled with GFP expression and this GFP expression may be sufficient to reveal axonal morphology within the CNS. The first task will therefore be to determine whether the central projections of sensory neurons can be visualised using the MARCM technique.

In order to ease the examination of sensory neuron central projections, individuals will be examined at the 3rd larval instar. No new sensory neurons are added to the embryonic complement during larval development so the same neurons will be present in third instar larvae as are present in the embryo. However, during larval development the size of the central nervous system increases dramatically. This should exaggerate the differences in central projection pattern between each individual sensory neuron and make any effects of ectopic

gene expression or loss of gene function on central projection pattern easier to detect. The normal pattern of central projections for each embryonic sensory neuron in the CNS of third instar larvae will therefore need to be established. This will provide a background against which the effects of a given gene on the growth of a particular sensory neuron can be measured. MARCM can then be used to examine the function of specific genes on the ordering of sensory projections within the central nervous system.

6.3 Materials and methods

6.3.1 Fly stocks

All fly lines were maintained at ambient temperature on standard *Drosophila* media (Appendix A). The MARCM stocks were obtained from Bloomington Stock Centre (Indiana, USA). Two different crosses were performed. For the first cross virgin females of the genotype P{neoFRT}19A, P{tubP-GAL80}LL1, P{hsFLP}1, w[*]; P{UAS-mCD8::GFP.L}LL5 were crossed to males of the genotype P{GawB}elav[C155] w[*] P{neoFRT}19A; Bc[1] Egfr[E1]/CyO to yield progeny of the genotype FRT GAL80 hs-FLP/elav-GAL4 FRT; UAS-CD8GFP/Bc or CyO. For the second cross virgin females of the genotype P{GawB}elav[C155], P{hsFLP}1, w[*]; P{>w[hs]>}G13 P{tubP-GAL80}LL2/CyO were crossed with males of the genotype y[1] w[*]; P{FRT(w[hs])}G13 P{UAS-mCD8::GFP.L}LL5 to yield progeny of the genotype elav-GAL4 hs-FLP/ y[1] w*; FRT UAS-CD8GFP/ FRT GAL80 or CyO.

6.3.2 Embryonic heatshocks

In order to collect embryos for heatshocking, crosses were allowed to lay for 1 hour at 25^{0}C on standard *Drosophila* media (Appendix A). Heat shocks were carried out at 0h, 1h, 2h, 3h, and 4h after laying by sealing the media plates with parafilm and immersing them in a water bath for 20, 30, or 40 minutes at 39^{0}C.

6.3.3 Screening of larvae for GFP expression in sensory neurons

After heatshock, animals were raised to the L3 larval stage. For detection of GFP expression in sensory neurons each larvae was anaesthetised using diethyl ether and placed under a coverslip in Drosophila Saline. Each larvae was screened individually for GFP expression using a Zeiss Axiophot II microscope equipped with epifluorescence.

6.3.4 Dissections

Dissections were performed in Drosophila Saline (Appendix A). For PNS preparations larvae were first cut open along the dorsal midline using extra delicate mini-Vannas dissecting scissors and the body wall was then pinned flat to a Sylgard dish (Sylgard Elastomer 184, Dow Corning) using minutien pins. All internal organs were removed and the PNS flushed

clean of any remaining extraneous tissues by gently blowing Drosophila Saline over the preparation using a pipette. At this point the CNS was removed and processed separately, when both the CNS and PNS were examined. The PNS was fixed in 4% paraformaldehyde for 10-30 minutes at room temperature and then processed for immunocytochemistry. Dissections of the CNS alone were performed by cutting the larvae in half using extra delicate mini-Vannas dissecting scissors and then turning the larvae inside out with a pair of size 4 forceps. The CNS was then detached from the body wall using a pair of fine (size 5) forceps and any extraneous tissues were removed. CNS preparations were fixed in 4% paraformaldehye at 4^0C overnight before being processed for immunocytochemistry (see below). Each PNS and CNS known to contain a sensory neuron clone was processed individually.

6.3.5 Immunocytochemistry

For CNS immunocytochemistry preparations were washed 2 x 15 minutes in PBS containing 0.4% triton-X (PBS-tx), washed in 2N HCL/PBS for 30 minutes, and then washed 4 x 15 minutes in PBS-tx before being incubated with Normal Horse Serum (Vector, UK) for 30 minutes (30ul of NHS in 1ml of PBS-tx). For PNS immunocytochemistry preparations were washed 4 x 15 minutes in PBS-tx before being incubated with Normal Horse Serum as above. Each PNS and CNS was incubated with a 1:200 dilution (5ul in 1ml PBS-tx) of anti-GFP primary antibody (Boehringer Mannheim) overnight at 4^0C. Before incubating overnight in a 1:200 dilution of secondary anti-mouse antibody (Vector, UK) (5ul in 1ml PBS-tx), each PNS and CNS was washed 4 x 15 minutes in PBS-tx. Preparations were then washed 4 x 15 minutes in PBS-tx, incubating for 2 hours in ABC solution (Vectastain ABC kit, Vector, UK) and washing a further 4 x 15 minutes in PBS-tx. To develop the reaction, preparations were transferred to 1 ml PBS-tx + 30 μl Diaminobenzidine solution (DAB; Sigma, UK) (final DAB concentration 0.3mg/ml) and reacted with 0.003% Hydrogen Peroxide (H_2O_2 ; Sigma, UK). Each CNS was pre-incubated for 15 minutes in DAB solution before developing the reaction. After the reaction was complete each preparation was washed a further 4 x 15 minutes in PBS-tx and then transferred to either 70% glycerol for mounting PNS preparations or brought through a series of 5 minute alcohol washes (30%, 70%, 80%, 90%, 3 x 100%), cleared in Xylene and mounted in Permount for CNS preparations.

6.4 Results

6.4.1 Visualisation of sensory axonal projections using the MARCM technique

While the MARCM technique has been used successfully to create tissue-specific mosaics within the nervous system, it was not known whether it could be used to label sensory projections within the CNS. As the cell body of a sensory neuron in the periphery could be located as much as two or three millimetres from the terminal projections within the CNS, would a sufficient quantity of GFP would be produced using the MARCM technique to reveal the entire sensory neuron axonal morphology?

To determine whether the MARCM technique could label sensory afferents it was initially decided to heatshock large numbers of embryos at set stages of development and then examine the CNS of all resulting 3rd instar larvae immunocytochemically for sensory neuron central projections. To aid the visualisation of sensory neuron central projections, the CD8-GFP fusion protein, which targets GFP to the plasma membrane, was used as a reporter gene (Lee and Luo, 1999). A large number of larvae could then be quickly screened for the presence of sensory neuron clones. Progeny of the genotype FRT GAL80 hs-FLP/ elav-GAL4 FRT; UAS-CD8GFP/ Bc or CyO were collected as embryos from 1 hour timed layings and heatshocked at either 0, 1, 2, 3 or 4 hours of development. Embryos were allowed to continue development until the 3rd larval instar and then dissected en masse and examined immunocytochemically for the presence of sensory neurons within the CNS.

Heatshocking embryos at either 0 or 1 hour after egg laying (AEL) resulted in near 100% mortality when assayed as 3rd instar larvae (data not shown) and those few larvae that survived were very small, slow to develop (approximately 8 days to reach the 3rd larval instar stage), and often showed external signs of abnormal development such as disorganised tracheal systems with degeneration of internal structures apparent upon dissection. When embryos were heatshocked at 3 hours AEL, neuroblast clones were observed within the CNS but no sensory neuron axonal projections were visible. This indicated that the chosen heatshock regime could successfully induce recombination at FRT sites on homologous chromosomes, causing the loss of the GAL80 repressor and subsequent activation of GFP expression in resulting clones. The frequently large size of the central neuroblast clones suggested that these clones were being induced at or near the beginning of neurogenesis and, as neurogenesis begins in both the sensory and the central nervous system at the same time, it seemed likely that heatshocking embryos 3 hours AEL should also induce sensory clones. In addition, neither

sensory nor central clones had been observed in the small number of embryos that had been heatshocked at 2 hours AEL.

More than 200 CNS' from 3rd instar larvae that had been heatshocked at 3 hours AEL were examined but no sensory projections were observed. Attempts were made to increase the sensitivity of the staining procedure by introducing an acid wash step in the protocol to improve antibody penetration and by increasing incubation times. A few faintly stained sensory projections were observed (3 out of more than 200) but were difficult to analyse due to the faintness of the signal. To optimise the quality of the material fixing tissues for both shorter and longer durations was experimented with but resulted in no significant improvements in quality (data not shown). I therefore decided to try and boost the GFP signal by setting up an alternative fly cross which would result in two copies of the GFP reporter gene being inherited by each clone upon loss of the repressor.

Progeny of the genotype elav-GAL4 hs-FLP/ y[1] w*; FRT UAS-CD8GFP/ FRT GAL80 or CyO were generated. As the parental FRT GAL80 insert is heterozygous, only 50% of progeny would be of the correct genotype. In order to ensure that sensory clones were indeed being induced after heatshocking progeny at 3 hours AEL, all larvae were screened individually using epifluorescence to check for the presence of sensory clones in the periphery. This screening showed that sensory neuron clones were present in approximately 10% of heatshocked progeny. Screening also enabled those progeny of the incorrect genotype to be eliminated, as these larvae did not contain the GAL80 repressor and therefore expressed GFP under the control of elav-GAL4 in all sensory neurons. When GFP expression within the CNS was examined immunocytochemically in those progeny containing sensory clones, the central projections of the GFP expressing sensory neurons were clearly visible. The MARCM technique can therefore be used to analyse sensory neuron central projections but two copies of the GFP transgene are required for reliable visualisation of the axonal morphology.

6.4.2 Generating sensory neuron clones using the MARCM technique

Embryos were heatshocked at 3 hours AEL to induce sensory neuron clones and allowed to continue development until the 3rd larval instar. All larvae were pre-screened using epifluoresence to identify and remove individuals that did not contain the GAL80 repressor. Each larva of the correct genotype was examined individually and all abdominal segments checked for the presence of GFP-expressing sensory neurons. This enabled any GFP-

expressing sensory neurons present to be identified and, as each CNS was processed individually for immunocytochemistry, ensured that one could unambiguously identify exactly which sensory neuron each central projection came from.

Over 1500 larvae have been screened individually so far, and 185 larvae were found to contain sensory neuron clones. Multiple clones within one individual were frequently observed. A summary of the numbers of clones recovered for each sensory neuron is given in Table 6.1.

Table 6.1 Summary of the number of clones recovered for each sensory neuron

Class of sensory neuron	Sensory neuron	No. of clones
ch neurons	vchA	5
	vchB	4
	v'ch1	4
	lch5.1	7
	lch5.2	7
	lch5.3	8
	lch5.4	8
	lch5.5	5
	lch?	20
md neurons	vda	25
	vbd	5
	vpda	3
	v'ada	11
	v'pda	5
	v'td2	10
	ldaA	3
	ldaB	7
	lbd	4
	ltd	10
	dbd	8
	dda	33
	dtd	2
es neurons	vesA	0
	vesB	0
	vesC	2
	v'esA	2
	v'esB	2
	v'es2	2
	v'es?	6
	lesA	4
	lesB/C	1
	desA2	1
	desC/D	1
	des?	12

Es neuron clones were recovered at a lower rate than other classes of sensory neuron, with ch neuron clones being most frequently generated. The observation of single md neuron clones in the periphery during the initial screening also revealed, firstly, that there are six md neurons in the dorsal cluster, one of which is associated with a tracheae and has therefore been classified as a tracheal dendrite neuron (Figure 6.3 C); and, secondly, that different md-da neurons can have significantly different and instantly identifiable dendritic branching patterns in the periphery. Some md-da neurons such as ddaE and ddaD in the dorsal cluster and vdaB in the ventral cluster are highly branched (Figure 6.3 A, B and Figure 6.4 C), with the dendritic field spanning the width of the segment. Others show highly restricted dendritic branching patterns in the periphery. In the dorsal cluster ddaB has its dendritic processes restricted to the anterior half of the segment (Figure 6.3 E) while ddaC projects only dorsally (Figure 6.3 D) and in the ventral cluster vdaA sends a single branch posteriorly until it reaches the denticle belt whereupon it forms profuse arborisations over the denticle belt (Figure 6.4 F). Additionally v'td and ltd were found to occasionally project anteriorly into the neighbouring segment (data not shown).

The axonal projections of sensory neuron clones within the CNS are shown in Figure 6.5. Visualisation of the axonal projections of each sensory neuron clone in the CNS is frequently complicated by the generation of clones within the ventral nerve cord itself. Such clones often contain neurons whose dendritic fields overlap the sensory projection and make it difficult to distinguish the axonal morphology of the sensory neuron from the dendritic branches of the central clone neurons (Figure 6.5 A). If a number of examples of the sensory neuron axonal morphology have already been determined, such preparations can often be interpreted or, as this analysis is ongoing, will be interpretable in the future when such examples are available. Additionally, multiple sensory neuron clones can occur within the same segment (Figure 6.4 B). In these cases while the axonal morphology of the sensory neurons may be clearly visible, it is impossible to determine which elements of the central projection pattern belong to which neuron unless the projection pattern has already been determined for each neuron in isolation.

To reduce the number of clones being generated, both to prevent central clones obscuring the sensory projection pattern and to prevent multiple independent sensory clones being generated in the same segment, heatshock durations of 30 minutes and 20 minutes were tested. With 20 minute heatshocks sensory clones were observed so infrequently (less than 1 larvae in 30) as to be impractical as it would be extremely time-consuming to screen so many larvae individually for so few clones, although the resulting larvae each contained only one sensory neuron clone. 30-minute heatshocks reduced the numbers of sensory clones within each

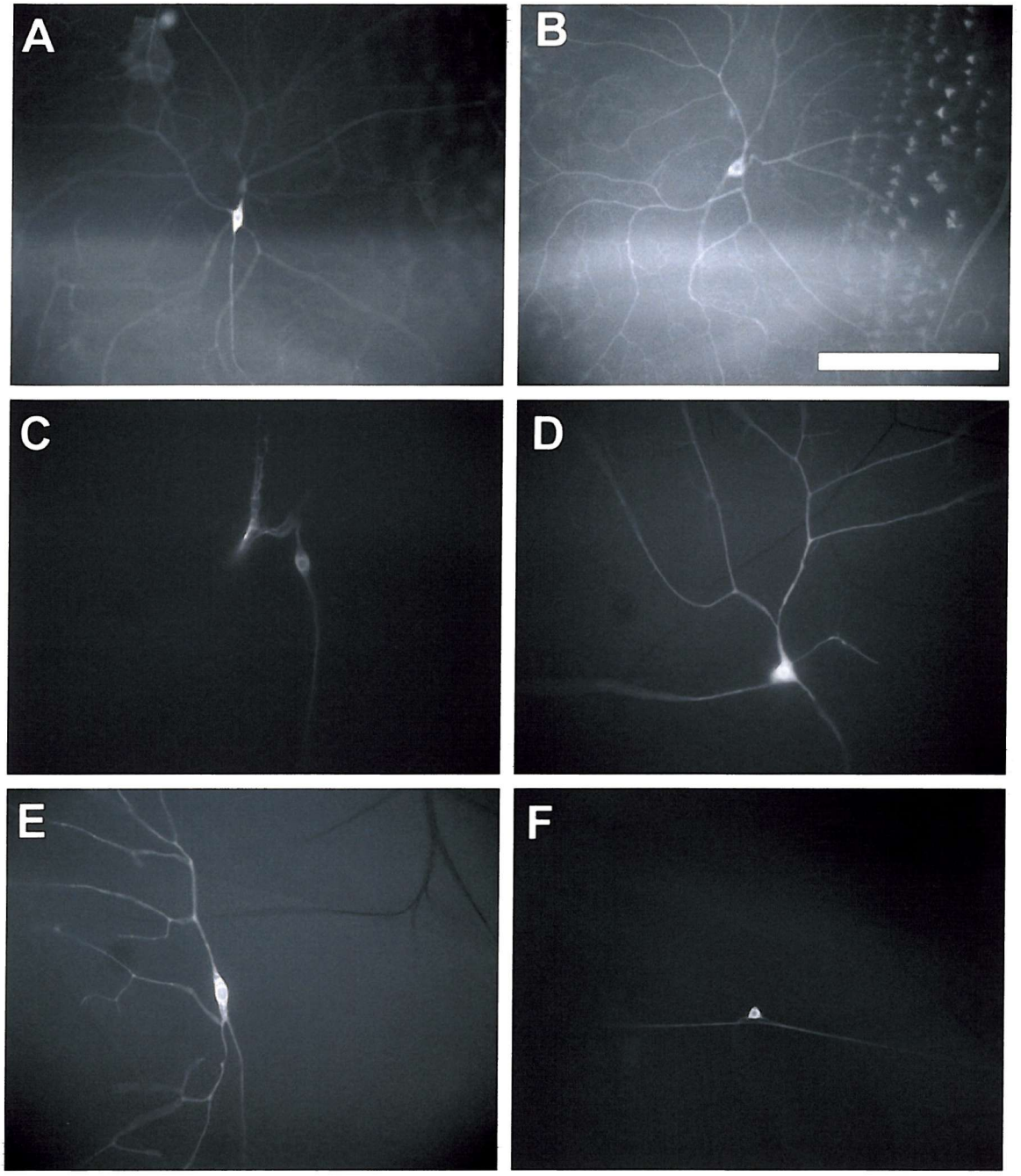


Figure 6.3 The dendritic branching patterns of dorsal cluster md neurons in the periphery. A. The dendritic arbours of ddaE, which are highly branched and span the width of the segment. B. The dendrites of ddaD are highly branched and span the width of the segment. C. The dorsal tracheal dendrite neuron, dtd. D. The dendrites of ddaC project dorsally only but span the width of the segment. E. The dendrites of ddaB are restricted to the anterior region of the segment. F. The dorsal bipolar neuron, dbd. The neurons have been named after the dorsal cluster neurons in the embryo although the designations of dorsal cluster md-das in the embryo are arbitrary, as they cannot be individually distinguished. (A-F) Scale bar 0.1mm

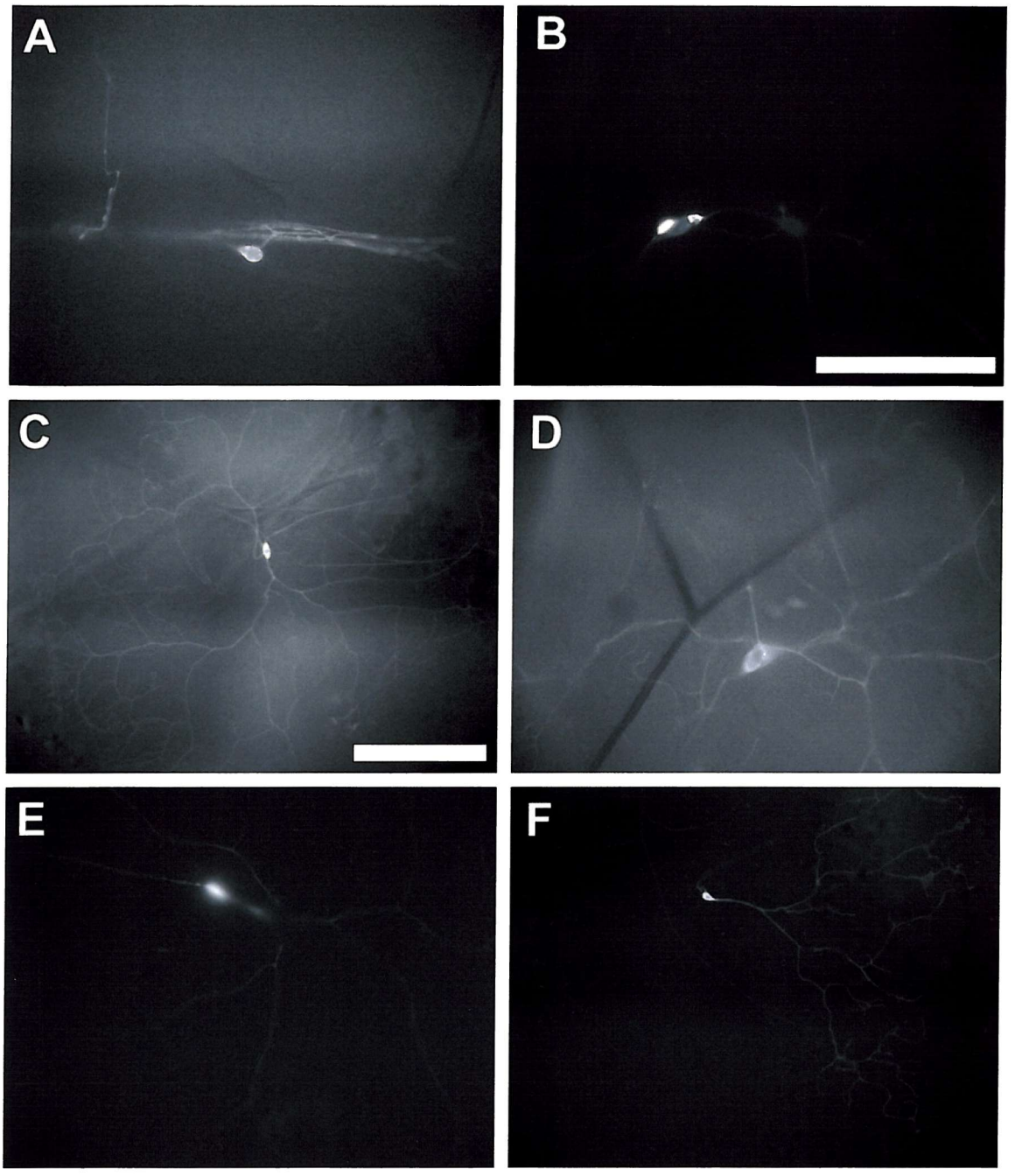


Figure 6.4 The dendritic branching patterns of ventral cluster md neurons in the periphery. A. The ventral bipolar dendrite neuron, vbd. **B.** Showing two sensory neuron clones that have been induced in close proximity within a single hemisegment. The neurons shown are lesC and v'pda. **C.** The dendrites of vdaB are highly branched and cover the width of the segment **D.** Shows the dendritic projections of vdaD, which cover the width of the segment. **E.** Shows the dendritic projections vdaC, which cover the width of the segment. **F.** Shows the posteriorly restricted dendrites of vdaA. The designations of ventral cluster md-das are arbitrary as the morphology of the ventral cluster changes slightly during larval development and so these neurons do not necessarily correspond to the similarly named neurons in the embryo. (A-B, D-E) Scale bar 0.1mm. (C, F) Scale bar 0.1mm

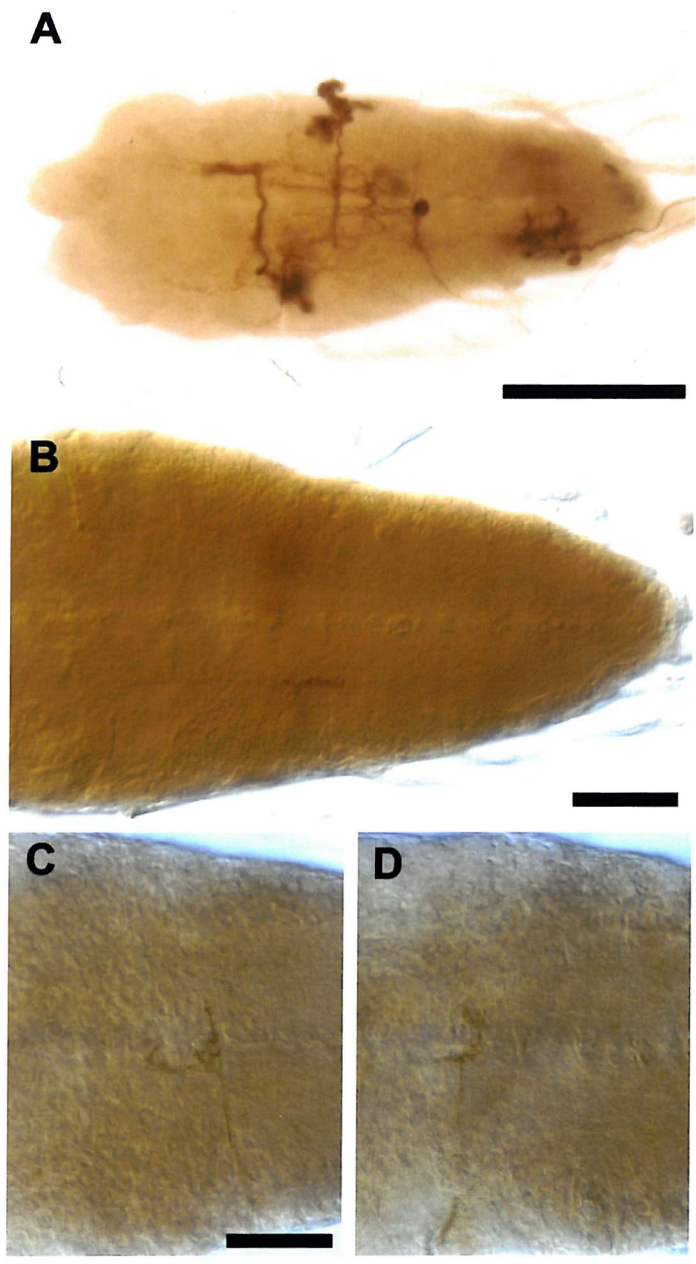


Figure 6.5 Sensory axons and central clones in the CNS of 3rd instar larvae. A. Example of a central clone generated in addition to sensory clones. **B.** The central projections of an lch5.3 chordotonal neuron. **C.** The central projection of the vdaC sensory neuron. **D.** The central projections of the vdaD sensory neuron. (A) Scale bar 0.6mm. (B) Scale bar 0.3mm. (C-D) Scale bar 0.3mm.

larvae while larvae containing sensory clones were still observed at an acceptable frequency (approximately 1 larvae in 12). Central clones that obscure sensory projections, and multiple clones within the same segment are still occasionally observed, although the 30-minute heatshock regime is a significant improvement over the 40-minute heatshock regime previously used.

6.4.3 Analysis of the central projections of sensory neurons in 3rd instar larvae

The characterisation of the central projections of embryonic sensory neurons in third instar larvae is not yet complete, due to time constraints, but the results obtained so far will be presented.

Each sensory neuron produces a characteristic pattern of central axonal projections and while there is some degree of variability in the projection of each neuron, the overall axon morphology is similar between individuals. Those groups of sensory neurons for which there is sufficient information available, such as the ch neurons and the md-das, show overall group features and tend to arborise within specific regions of the neuromere, enabling most neurons to be instantly recognisable as belonging to a particular class and in many cases to also identify the individual sensory neuron. While this analysis is ongoing, preliminary results for each class of sensory neuron will now be presented.

Chordotonal neurons

Ch neurons characteristically have lengthy longitudinal axonal processes which lie within a discrete lateral tract (Figure 6.6). The axon enters the CNS and upon reaching the lateral tract splits to form an anterior and a posterior process. The extent of the processes in the anterior and posterior directions is variable and depends upon neuronal identity. Many subbranches extend from the main longitudinal process and these are primarily restricted to the lateral tract. Within this lateral tract there is a trend for a progressive lateral to medial shift in axon distribution that corresponds with a ventral to dorsal shift in location of the neuron on the body wall. A preparation containing both a dorsal md-da neuron and a lateral chordotonal neuron that has been mounted side-on indicates that the axons of ch neurons occupy a medio-ventral position within the neuropil (Figure 6.7).

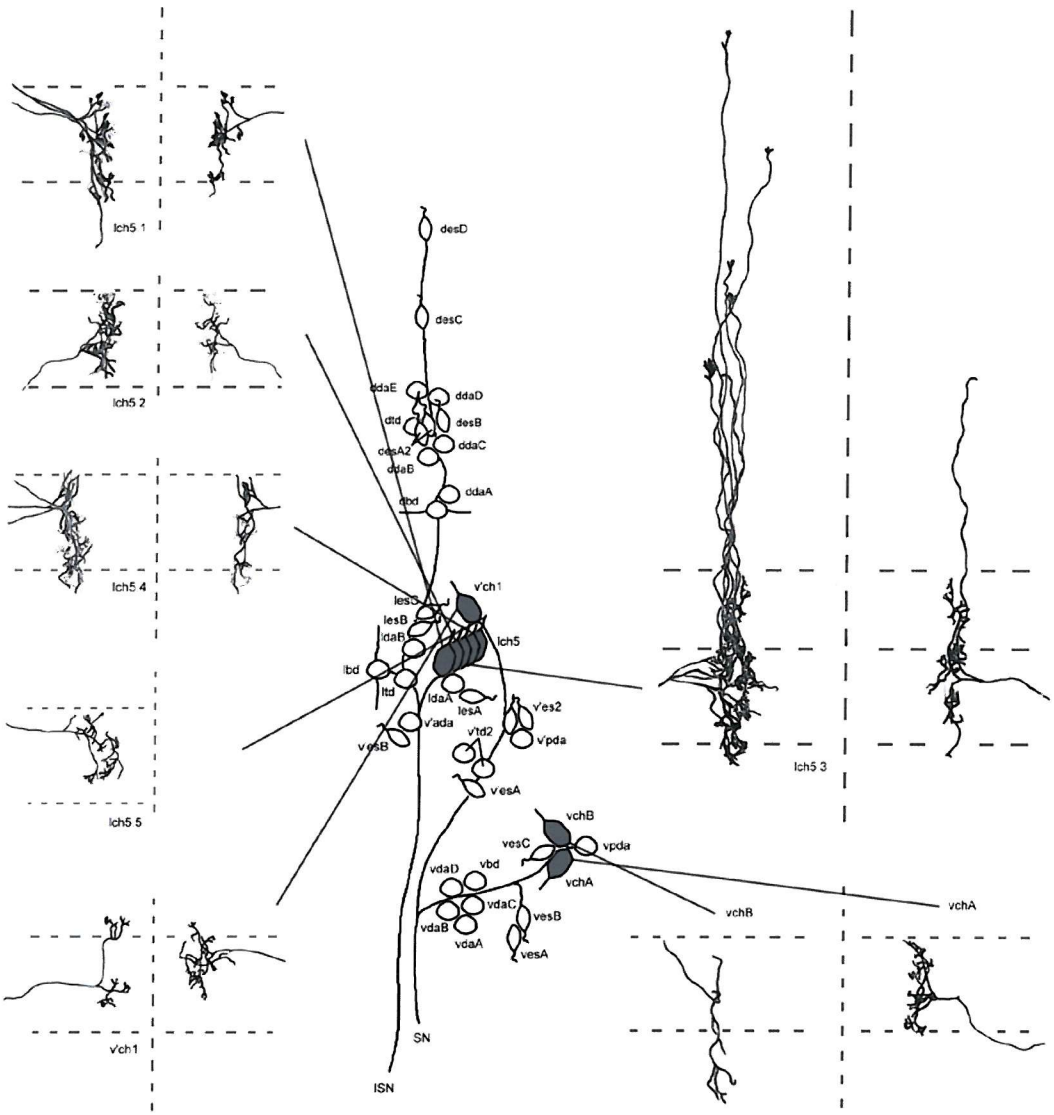


Figure 6.6 The central projections of the chordotonal neurons. Anterior is up in this and all subsequent figures. A vertical dotted line indicates the position of the midline and horizontal dotted lines indicate segment boundaries. The positions of the ch neurons are shown in the central panel. For neurons where multiple examples of the CNS morphology were available the left side shows the central projections of individual neurons superimposed while the right shows a single representative neuron. Width of average neuromere 0.3mm.

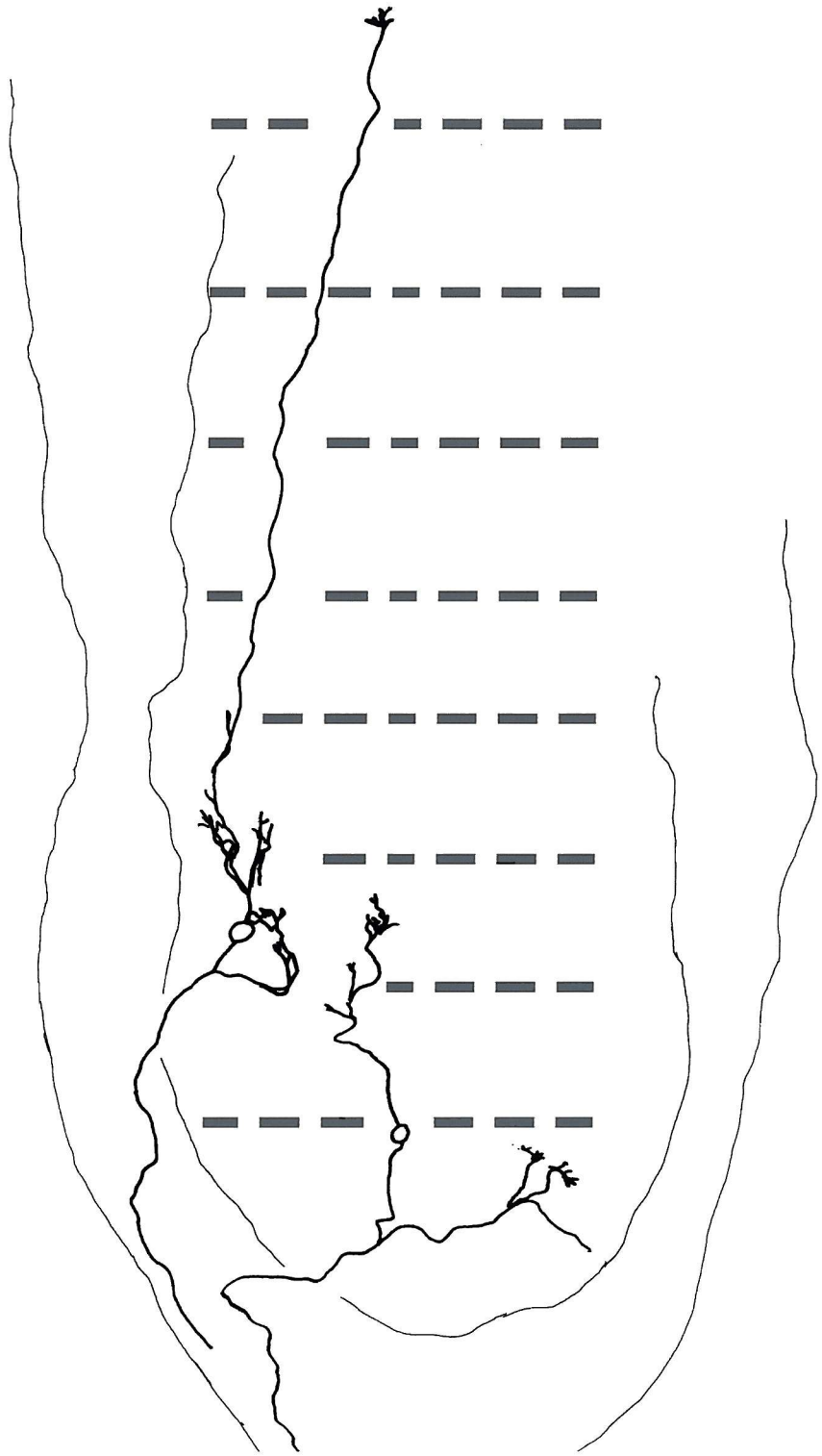


Figure 6.7 Lateral view of sensory neuron central projections. The lch5.3 neuron occupies a much more ventral position within the neuropile than the dorsal md-da neuron. The figure also shows that the dorsal md-da produces both a medial branch and a dorsally projecting branch. Anterior is up and dorsal is to the right.

lch5.1

Axons form longitudinal processes within the lateral tract at its medial-most edge (Figure 6.6). A posterior process extends into the adjacent neuromere and a shorter, anteriorly directed process extends to the segment boundary. n=3

lch5.2

Axons form anterior and posterior longitudinal processes within the lateral tract that extend the width of the segment (Figure 6.6). Subbranches extend the width of the lateral tract and occasionally project slightly outside the lateral tract towards the midline. One lch5.2 neuron in A7 entered the CNS via the A7 nerve and projected through the cortex to A5, where it entered the neuropil and formed a typical lch5.2 projection. n=4

lch5.3

Axons form an anterior process than extends through multiple neuromeres and a comparatively shorter posterior process than extends into the adjacent posterior neuromere (Figure 6.6). The anteriorly directed process frequently terminates in the thoracic neuromeres and can extend through as many as 6 neuromeres to terminate in the thorax (clones located in A5. n=3). Six neuromeres could be the upper limit on axon extension for lch5.3 neurons as the anterior process of an lch5.3 clone located in A7 terminated in A2. Not all clones terminate in the thoracic neuromeres although all extend anteriorly for several neuromeres. Those clones that do extend into the thorax tend to terminate in T3 (n=4). In one case the opposite morphology was observed. A single lch5.3 neuron in A2 extended one neuromere anteriorly to terminate in A1 neuromere and three neuromeres posteriorly to terminate in A5 but was in all other manners consistent with the lch5.3 morphology. n=7

This unique ch neuron morphology, where an anteriorly directed process is present that extends through several neuromeres, was previously attributed to lch5.4 in first instar larvae (Schrader and Merritt, 2000). However because of the proximity of the ch neuron cell bodies within the lateral pentascolopale it is difficult to accurately determine which neuron was visualised in this analysis (D. Merritt, personal communication). The identification of individual ch neurons within the lch5 organ in living third instar larvae, based on position within the pentascolopale and position of the axon and the spur in relation to the cell body

which is different for each lch5 neuron (L. Block, unpublished observations), is therefore likely to be more accurate and this neuron is likely to be lch5.3 rather than lch5.4.

lch5.4

Most axons (n=4) form a posterior process that extends into the neighbouring segment and a shorter anterior process (Figure 6.6). However an lch5.4 neuron in A2 (n=1) showed the opposite morphology, with the bulk of the projection being in the anterior direction. The overall axonal morphology of the A2 lch5.4 neuron was consistent with the general characteristics of the lch5.2 projection, albeit in reverse. Additionally an lch5.4 neuron in A7, which entered the CNS via the appropriate peripheral nerve, was observed to enter the neuropil in A5, rather than A7, where it formed a typical lch5.4 projection. n=5

lch5.5

There is only one clear example of the lch5.5 axonal morphology so far. The axon appears to have shorter anterior and posterior processes and spans a wider area in the medio-lateral axis than the other lch5 neurons (Figure 6.6). The axon makes a short anteriorly directed process and a longer posteriorly directed process within the lateral tract and also makes a second, more medial, posteriorly directed process just outside the lateral tract. It is not clear whether this projection is representative of the lch5.5 axonal morphology although it does appear superficially similar to the lch5.5 morphology in the embryo (Merritt and Whitington, 1995).

vchA

There is just one clear example of the vchA axonal morphology. The longitudinal processes extend both anteriorly and posteriorly and span the width of the segment (Figure 6.6). Subbranches span almost the width of the lateral tract.

vchB

Only one clear example of the vchB projection has been recovered so far. The axon splits to form a lengthy posterior and a shorter anterior process at the lateral-most edge of the lateral tract (Figure 6.6). The posteriorly directed process extends into the neighbouring neuromere. The vchB projection is relatively unbranched compared to the other chordotonal neurons.

v'ch1

There are two clear examples of the v'ch1 axonal morphology. The primary longitudinal process lies just medial to the lateral tract and extends both anteriorly and posteriorly, with the anterior process crossing the segment boundary (Figure 6.6). While both examples span a similar distance in the anterior-posterior axis, one show significantly more subbranches than the other and, until more clear examples have been described, it is unclear which neuron provides a truer representation of the v'ch1 central projection pattern.

External sensory neurons

Not enough es neuron central projection patterns have yet been resolved to define a characteristic es neuron axonal morphology as in only four preparations can the central projection pattern be unambiguously determined. These are shown in Figure 6.8, but it is not yet known whether each of these neurons is truly representative of its class. Nevertheless, it is already clear that the central projections of es neurons are not restricted to a longitudinal tract. They show less extensive subbranching than either ch or md neurons. In addition, none of the es neurons so far identified send processes across the midline, which is consistent with the embryonic descriptions of these es neurons (Merritt and Whitington, 1995)

vesC

The vesC axon forms a single unbranched process which extends medially and anteriorly towards the segment boundary and continues into the neighbouring neuromere where begins to extend in a more lateral direction (Figure 6.8).

v'esA

The axon splits to form an anteriorly directed and a posteriorly directed process (Figure 6.8). The anterior branch projects to the midline but does not cross and extends longitudinally into the neighbouring neuromere. The posterior branch projects to the midline and splits again to extend along the midline in both the anterior and posterior directions.

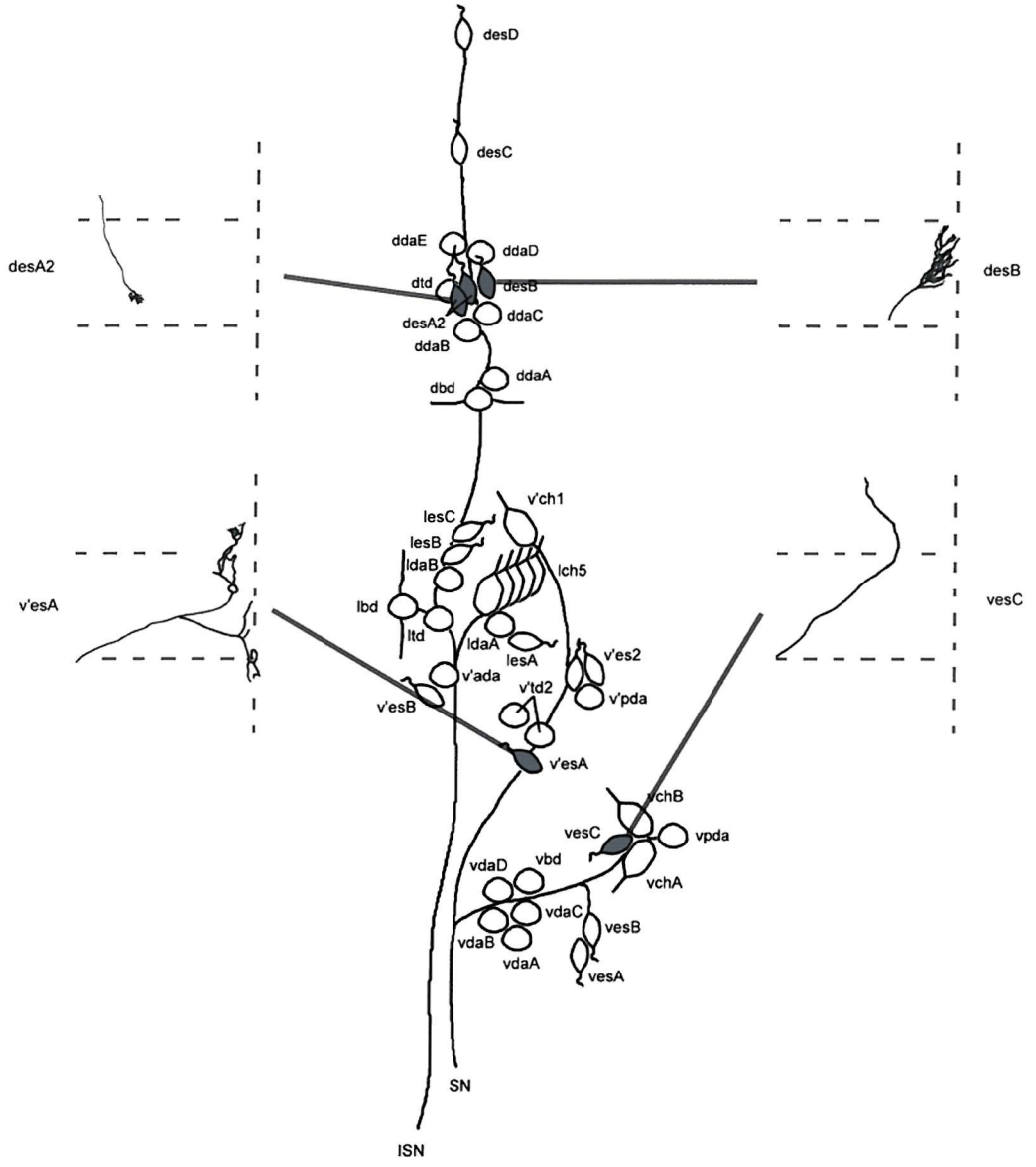


Figure 6.8 The central projections of the es neurons. A vertical dotted line indicates the position of the midline and horizontal dotted lines indicate segment boundaries. The positions of the es neurons are shown in the central panel. A single example of each es neuron is shown. Width of average neuromere 0.3mm.

desA2

The axon terminates at a lateral position within the neuromere and forms a small number of subbranches at this point (Figure 6.8). No branches extend from the main axon prior to this point.

desB

The axon extends to the midline but does not cross and continues along parallel with and close to the midline in an anterior direction until it reaches the segment boundary (Figure 6.8). Numerous medially located subbranches extend to the midline in the anterior half of the neuromere.

Multidendritic neurons

The md neurons are subdivided into three categories according to their dendritic morphology: md-da neurons that produce dendritic arbours that ramify beneath the epidermis, md-td neurons whose dendrites are associated with trachea, and md-bd neurons which possess bipolar dendrites. From the analysis of their dendritic arborisation patterns in the periphery the md-da neurons can be further subdivided according to their dendritic morphology into md-das that produce dendritic arbours over the whole width of the segment and md-das that have their dendritic arbours restricted to different regions of the segment (Figures 6.3 and 6.4). The axonal projections of these neurons broadly reflect these divisions.

Md-da

These axonal morphologies can be divided into different groups and these groups are correlated with the dendritic arborisation patterns produced by each neuron in the periphery.

Of the dorsal cluster and ventral cluster md-das, vdaA is the only neuron which does not project towards the midline, instead forming an anteriorly directed process in a lateral position which extends into the neighbouring segment (Figure 6.9). A number of subbranches are evident but are also restricted to this lateral region of the neuromere. vdaA also produces a unique pattern of dendritic arbours in the periphery, extending a single branch posteriorly towards the denticle belt, which then produces branches over the entire area of the denticle belt (Figure 6.4 F). vdaA may therefore form a separate group from the rest of the md-das.

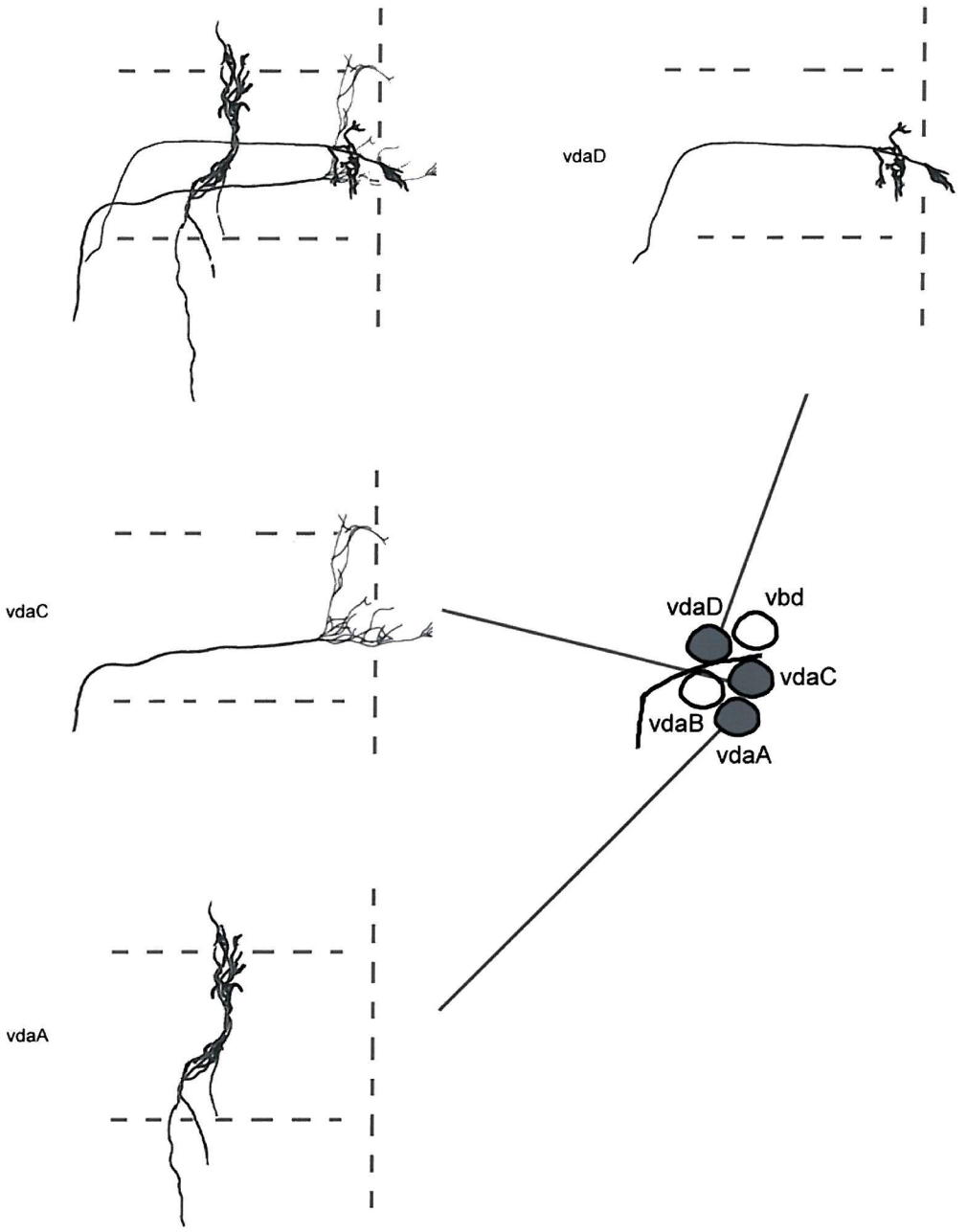


Figure 6.9 The central projections of the ventral cluster md-da neurons. A vertical dotted line indicates the position of the midline and horizontal dotted lines indicate segment boundaries. The positions of the md-da neurons are shown in the central panel. The central projections of the different ventral-cluster md-da neurons are show individually and superimposed (top right). Width of average neuromere 0.3mm.

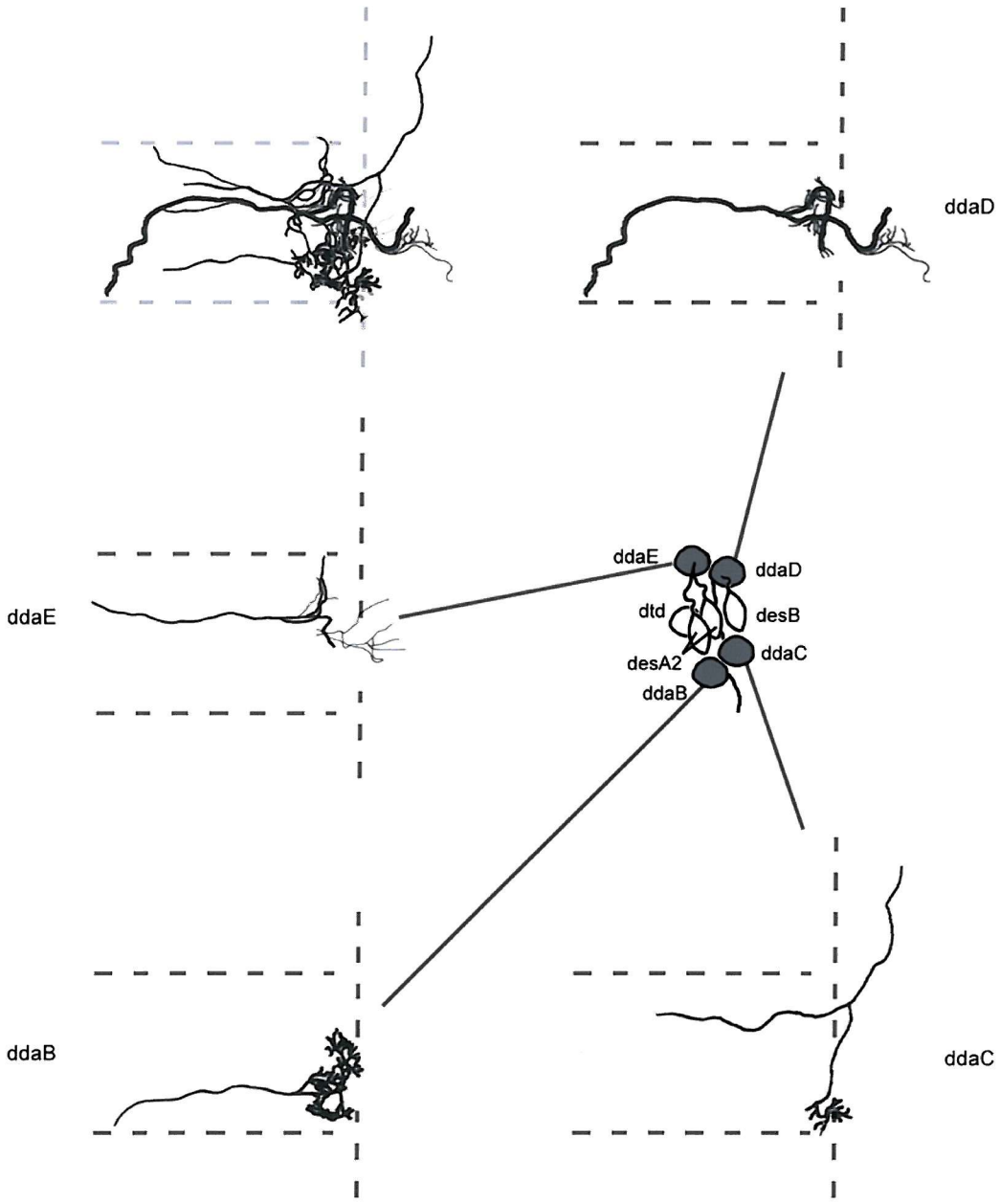


Figure 6.10 The central projections of the dorsal cluster md-da neurons. A vertical dotted line indicates the position of the midline and horizontal dotted lines indicate segment boundaries. The positions of the md-da neurons are shown in the central panel. The central projections of the different dorsal-cluster md-da neurons are show individually and also superimposed (top right). Width of average neuromere 0.3mm.

All other md-das of the dorsal and ventral cluster form medially located projections and have dendrites which arborise across a significant region of the segment in the periphery. With the exception of ddaB, they all produce an axonal process which crosses the midline and ddaB can be separated from the rest of the remaining ventral and dorsal md-das in that its dendrites are restricted to the anterior half of the segment in the periphery (Figure 6.9 and 6.10). All md-das whose dendrites ramify throughout the segment in the periphery cross the midline in the middle of the segment and terminate on the ipsilateral side. ddaE and vdaC in particular, which both produce extensive dendritic arborisations throughout the segment in the periphery, show a very similar axonal morphology within the CNS with both axons producing two main processes, one of which extends anteriorly to the segment boundary and one of which extends across the midline to terminate on the opposite side.

The dorsal cluster md-da ddaC shows a slightly different dendritic morphology in the periphery, where its dendrites are restricted dorsally (Figure 6.3 C), and also shows a different axonal morphology in the CNS (Figure 6.10). It does not produce any processes on the ipsilateral side of the CNS prior to crossing the midline. Upon crossing the midline however, the axon splits to produce an anteriorly directed process, which extends into the neighbouring neuromere, and a posteriorly directed process, which recrosses the midline to terminate on the ipsilateral side at the posterior segment boundary. Very few subbranches are present, and these are restricted to the posteriorly directed process on the ipsilateral side.

The lateral md-das ldaA and ldaB and the ventro-lateral md-da v'ada form projections that are morphologically distinct from the central projections of the dorsal cluster and ventral cluster md-das (Figure 6.11). These projections occupy a more lateral position within the neuromere and do not project to or cross the midline. The projections of both lateral md-das do not extend the width of the segment. The ldaA neuron produces two anteriorly directed processes in close proximity that lie parallel to each other. A number of small subbranches are present along the length of both anterior processes. The ldaB axon projects medially, producing two small anteriorly directly processes and then extends a posterior process and a smaller anterior process. Numerous subbranches are present along these processes. The ldaB projection is more medial than the ldaA projection, while the v'ada projection occupies a more lateral position within the segment than the lateral md-das. The v'ada axon forms an anteriorly directed laterally located projection that spans the width of the segment and is highly branched.

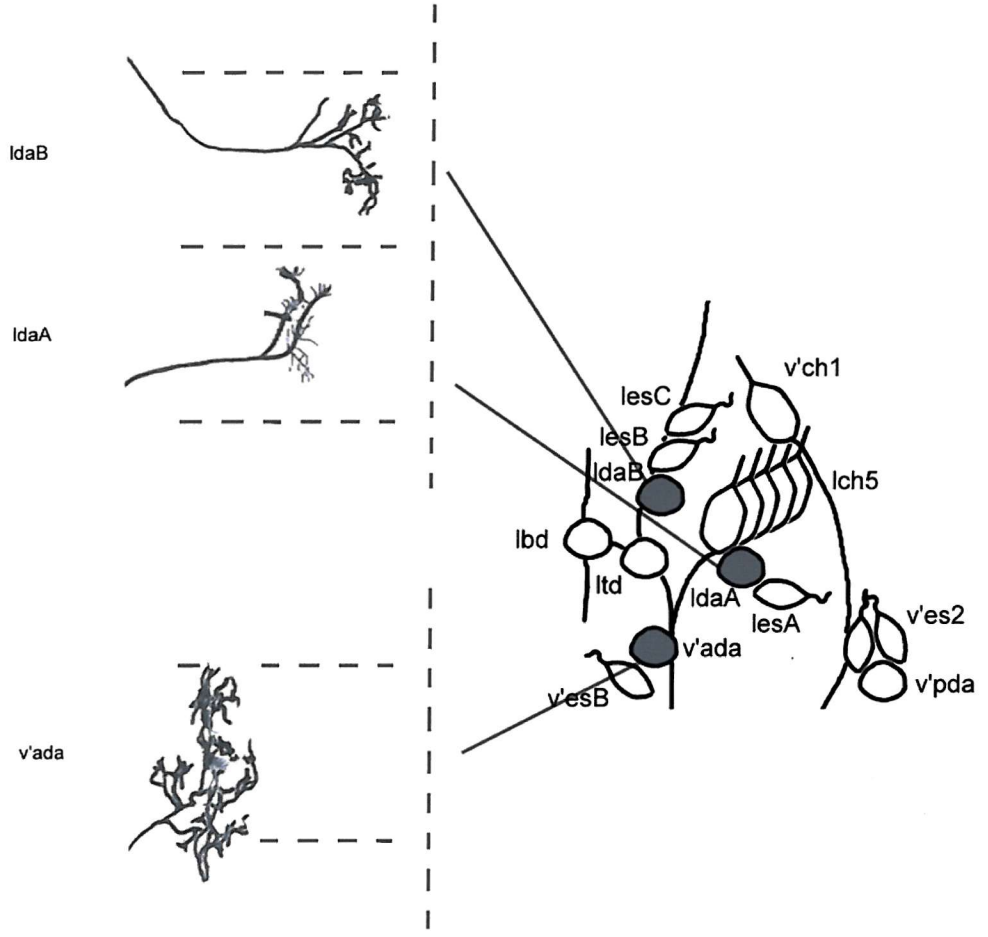


Figure 6.11 The central projections of the lateral and ventro-lateral md-da neurons. A vertical dotted line indicates the position of the midline and horizontal dotted lines indicate segment boundaries. The positions of the md-da neurons are shown in the central panel. The central projections of the different md-da neurons are show individually. Width of average neuromere 0.3mm.

Md-bd

There is only one clear example of the dbd axonal morphology but extrapolation from the partially obscured morphology visualised in other preparations suggests this may be representative. The dbd neuron forms a distinctive axonal morphology close to the midline, which is relatively compacted compared to other sensory neurons and spans approximately half the width of the segment (Figure 6.12). This is consistent with the shortening of the axon terminals from the embryonic projection pattern observed in first instar larvae (Schrader and Merritt, 2000). While no unambiguous preparations containing vbd have yet been recovered, a single lbd axonal projection has been visualised. The central projection formed by the lbd neuron has not previously been described at any stage in development. It occupies a far more lateral position within the neuromere and is far less compacted than the dbd axon, with numerous subbranches visible, although again occupies approximately half the width of the segment. The morphology and position of the lbd neuron is similar to the lateral and ventro-lateral md-da neurons ldaA, ldaB and v'ada and more similar to these neurons than to dbd.

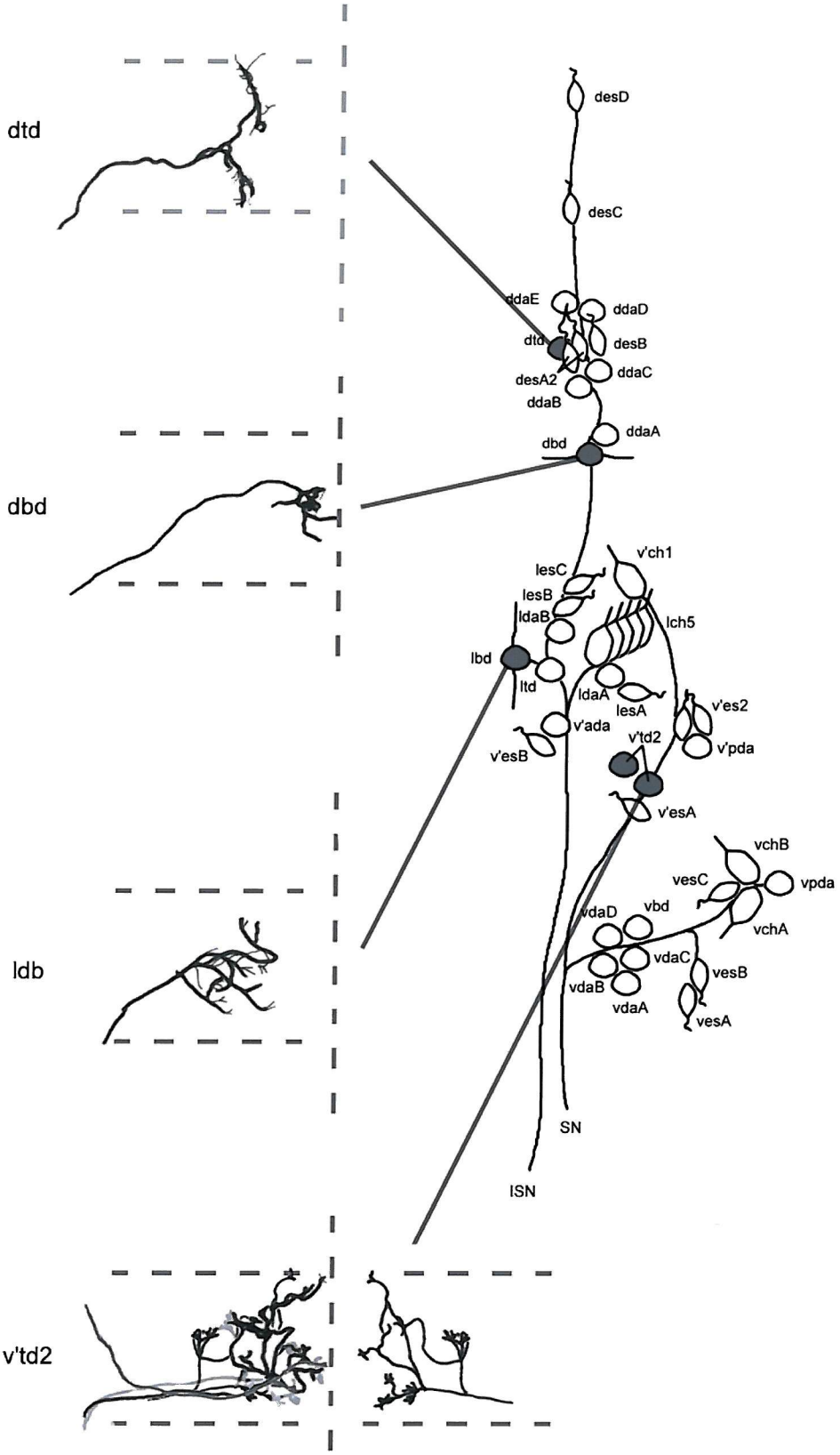
Md-td

There are four trachea-associated sensory neurons in each abdominal hemisegment. Central projection patterns have been unambiguously determined for the newly identified dorsal md-td neuron, which I have named dtd, and for v'td neurons but not for the ltd neuron.

The dtd neuron occupies a medio-lateral position within the neuromere (Figure 6.12). It projects medially past the lateral tract occupied by the chordotonal neurons and then extends both anteriorly and posteriorly to the segment boundaries, producing processes of approximately equal length in both directions. Small subbranches extend from the main processes in all directions.

Each v'td2 neuron could not be individually identified when visualised in isolation in the periphery and it is unclear whether the observed sensory projection patterns belong to more than one neuron. Nevertheless, most axons entered the CNS and projected towards the midline with a lateral process and a medial process branching off in an anterior direction from the main medially directed axon, which terminates close to the midline. The lateral branch extends anteriorly for approximately half a segment before terminating while the lateral branch extends anteriorly and medially to terminate at the segment border close to the midline. There is some variation in the number and extent of subbranches present but most axons conform to

Figure 6.12 The central projections of the md-td and md-bd neurons. A vertical dotted line indicates the position of the midline and horizontal dotted lines indicate segment boundaries. The positions of the md neurons are shown in the central panel. For dtd, dbd, and lbd, a single example of the central projection pattern is shown. For v'td2 the left side shows the central projections of individual neurons superimposed while the right shows a single representative neuron. Width of average neuromere 0.3mm.


dtd
dbd
ldb
v'td2
desD
desC
ddaE
ddaD
dtd
desB
ddaC
desA2
ddaB
ddaA
dbd
v'ch1
lesC
lesB
ldaB
lch5
lbd
ltd
ldaA
lesA
v'es2
v'ada
v'esB
v'td2
v'pda
v'esA
vchB
vesC
vpda
vbd
vdaD
vchA
vdaC
vesB
vdaB
vdaA
vesA
SN
ISN

this general morphology (n=4). The v'td neurons have previously been described to show unusual and segment specific axonal morphologies within the CNS, with the axons extending anteriorly in a very lateral fascicle to terminate in T3 (Merritt and Whitington, 1995) but all preparations included here arborised within their segment of origin. All preparations included in the original analysis in the embryo seem to have been taken from A1-A3 whereas two of the clones included here originated in A5 and A6. An additional v'td2 clone in A1 was also observed but, while the central projection pattern was clear, damage to the surrounding CNS prevented complete visualisation of all neuromeres and the neuromere in which this neuron made its projection could have been misidentified as A1. A fourth clone within A3 also formed its projection within its own segment. It remains possible that the two v'td2 neurons show divergent morphologies and that all clones described here are produced by only one of these v'td2 neurons. Alternatively the central projection pattern described here may be a larval specific element of the central projection pattern and a second part of the projection that did extend laterally to T3, may not have been clearly visualised. In support of this, a process that branches off from the main axon near its entry point into the neuropil has been described in some individuals for the v'td2 neurons in first instar larvae (Schrader and Merritt, 2000). Additional data will be required to resolve this discrepancy.

6.5 Discussion

The work presented in this chapter has demonstrated that the MARCM technique can be used to reveal the central projection pattern of sensory neurons. While the characterisation of sensory neuron central projections in third instar larvae is ongoing, the preliminary results of this analysis have been presented. When complete, this should provide a framework that enables gain of function and loss of function analysis of the effects of candidate genes on determining axonal morphology to be conducted. Preliminary findings show that each neuron produces a specific pattern of central projections and that the greater spatial resolution afforded by the CNS of the third instar larvae does indeed exaggerate the differences between each neuron. This has shown that the central projections of different groups of sensory neuron can be defined by specific features and the position of their processes within the neuromere, and that individual sensory neurons can also be identified by their projection pattern. In addition, examination of individual md-da neurons in the periphery revealed that the different md-das produce distinct patterns of dendritic arbours, and these differences in dendritic morphology in the periphery are also reflected in the axonal morphology of these neurons within the CNS. Insufficient es neurons have yet been described to determine a characteristic es neuron projection and these will not be discussed further.

The CNS of the third instar larvae should therefore provide an ideal system in which to examine the effects of specific genes on central projection pattern.

6.5.1 Central projections of chordotonal neurons

As in the embryo, all ch neurons project within a distinct lateral tract with the exception of v'ch1, which in the larva forms its projection just outside the medial edge of the lateral tract. Each individual ch neuron forms a characteristic projection pattern within the CNS that is uniquely identifiable. The ch neuron lch5.3 produces a particularly unique morphology where it extends anteriorly through several neuromeres, frequently terminating in the T3 thoracic neuromere. While the morphology of the individual ch organs in the lateral pentascolopale is indistinguishable in the periphery, the different axonal projections produced by each neuron in the CNS, particularly lch5.3, suggests that even in such an apparently uniform organ individual neurons may have undergone some degree of functional specialisation. Different chordotonal receptor cells within the locust metathoracic femoral chordotonal organ show different response types, with different receptors responding to position, velocity, position and velocity and also more complex stimuli, possibly including acceleration (Matheson, 1990).

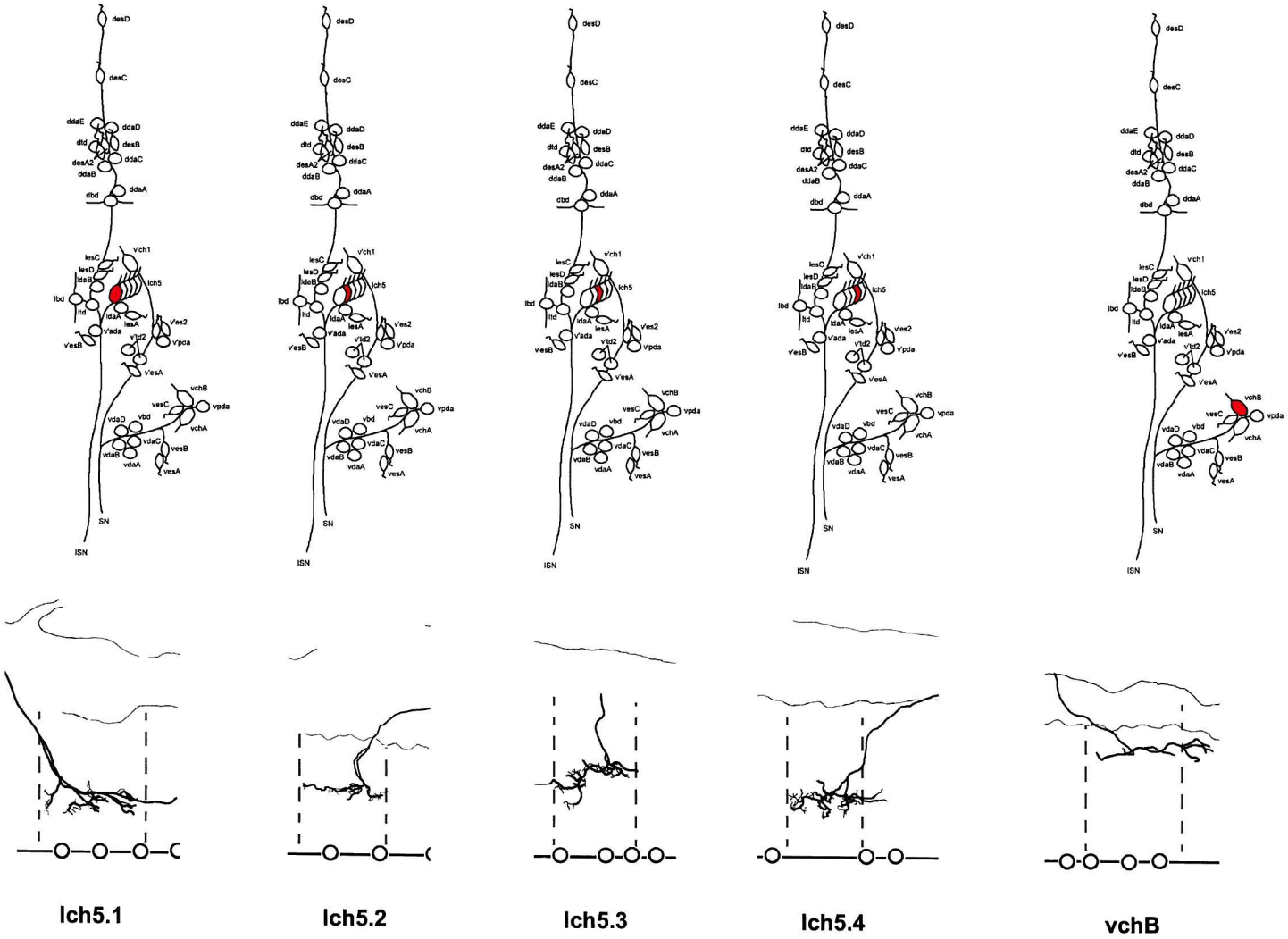
Interestingly, receptors with comparable responses also had cell bodies in similar locations and dendrites with similar orientations (Matheson, 1990). Different receptors within the lateral pentascolopale may also respond to different stimuli. As the lch5.3 neuron projects through multiple neuromeres, it could potentially play a role in the co-ordination of movement between segments.

A number of unusual features were also noted in certain ch neuron projections. A reversed axonal morphology, where the bulk of the longitudinal processes extends in the opposite direction from that observed for all other neurons of that type, was observed in a single lch5.3 neuron and a single lch5.4 neuron. Both clones were located in abdominal segment A2, suggesting that there could be segment specific changes in axon morphology. However, as these are only two ch neuron clones in A2, there is currently insufficient data to substantiate such a claim. Also, on two occasions, ch neuron clones located in A7 entered the CNS via the appropriate A7 nerve and then projected through the cortex to A5, where they entered the neuropil and formed the appropriate projection, albeit in A5 rather than A7. A further two ch clones in A7 entered the neuropil and formed their projection pattern in A7, suggesting that this is not a general rule for ch neurons in A7 and also not a property of specific ch neurons, as one lch5.2 clone in A7 entered the neuropil in A7 while another entered the neuropil in A5. As far as I am aware, there are no other examples of sensory neurons that do not enter the neuropil in their segment of origin. The only conceivable candidates are the v'td neurons that were found in the embryo to project along a very lateral fascicle to terminate in T3, regardless of segment of origin (Merritt and Whitingdon, 1995), as it is possible, but unlikely, that with the limited resolution afforded by the embryonic CNS, any projection though the cortex would not have been distinguished from projection through a very lateral fascicle.

6.5.2 Do chordotonal neurons produce somatotopically ordered central projections?

While no obvious somatotopic projection was discernible in the embryo (Merritt and Whitingdon, 1995), the greater spatial resolution provided by the CNS of 3rd instar larvae reveals that there is a distinct trend in the projections of ch neurons. Neurons that are located more ventrally on the body wall tend to project more laterally within the lateral tract while neurons that are located more dorsally on the body wall tend to project more medially within the lateral tract with v'ch1, which is the most dorsally located ch neuron in larvae forming the most medial projection of all, just outside the lateral tract. This is shown in Figure 6.13. The correspondence is not exact however, as while there is a progressive shift from medial to lateral in the projections of lch5.1 to lch5.4, lch5.5, which is the most ventral of the larval lch5

Figure 6.13 Summary of chordotonal neuron central projections. The central projections of individual ch neurons and their peripheral location are shown. There is a trend for axons to project more medially as the location of the ch neuron cell body in the periphery shifts from ventral to dorsal. Anterior is left and medial is down. Broken lines indicate segmental boundaries. Unbroken lines indicate midline. Circles represent midline cells.


desD
desC
ddaE
ddaD
dtd
desB
desA2
ddaC
ddaB
ddaA
dbd
lesC
lesD
ldaB
lch5
lbd
ltd
ldaA
lesA
v'ada
v'es2
v'esB
v'td2
v'pda
v'esA
vchB
vesC
vpda
vdaD
vbd
vchA
vdaC
vesB
vdaB
vdaA
vesA
SN
ISN
lch5.1
lch5.2
lch5.3
lch5.4
vchB

neurons and should therefore form the most lateral projection within the lateral tract of the lch5 neurons, forms a projection at a medial position within the lateral tract and also projects medially outside the lateral tract. Only one clear example of the lch5.5 morphology has been recovered so far so it is unclear whether the projection described is representative of the lch5.5 projection. Again, while vchB, the second-most ventrally located ch neuron, projects to the lateral-most edge of the lateral tract, vchA, the most ventrally located ch neuron, projects to a comparatively medial location within the lateral tract and produces processes that span much of the lateral tract. The projections of both vchA and vchB are drawn from a single individual and further examples of their axonal morphology will be required.

6.5.3 Central projections of dendritic arborisation neurons

The md neurons are divided into three classes based on their dendritic morphology which are the md-td neurons, the md-bd neurons and the md-da neurons, which are considered to be a homogenous group. The examination of single md-da clones in third instar larvae revealed that different md-das have significantly different and instantly identifiable dendritic branching patterns in the periphery and can be further subdivided into different subclasses based on this morphology: md-das that have extensive dendritic arborisations in all directions and that span the width of the segment, md-das whose dendrites are restricted to the anterior half of the segment, md-das whose dendrites are restricted to the dorsal region above the cell body, and an md-da that only produces dendritic arbours over the denticle belt. These different dendritic projection patterns could reflect the subdivision of the md-das into different functional groups, as has been shown for dendritic arborisation neurons in *Manduca sexta* (Grueber and Truman, 1999).

The central projection patterns of the md-da neurons can also be divided into different groups and these groups are correlated with their dendritic morphology in the periphery. The md-das that arborise extensively form medially located projections and produce a branch that crosses the midline and terminates contralaterally. The dorsal md-da whose dendrites project anteriorly forms a medially located projection but does not cross the midline, while the dorsal md-da whose dendrites project dorsally does not produce any processes on the ipsilateral side of the CNS prior to crossing the midline and upon crossing the midline extends anteriorly into the neighbouring neuromere and posteriorly back across the midline to terminate on the ipsilateral side. The md-da that arborises over the denticle belt forms an anteriorly directed process in a lateral position, which extends into the neighbouring segment. The axonal projections of the lateral md-das, which occupy a more lateral position within the neuropil are

distinct from the ventral and dorsal md-das, although they do show a high degree of dendritic branching in the periphery. Each group therefore produces a distinct axon morphology and frequently projects to a different region of the neuropil, providing further support for the notion that md-das are subdivided into different functional groups.

6.5.4 Do the central projections of dendritic arborisation neurons show evidence of any underlying somatotopic organisation?

There is no direct correlation between position on the body wall and the lateral to medial or anterior to posterior extent of the md-da projections in the CNS. When axonal morphology is compared with peripheral pattern of dendritic arborisations however, a possible relationship becomes apparent. While the dorsal and ventral md-das that produce extensive dendritic arbours have medially located central projections that cross the midline, the lateral md-das which also have expansive dendritic arborisations do not. Their central projections occupy a more lateral position and do not approach the midline. All dorsal and ventral md-das that cross the midline in the CNS also have dendrites that branch towards the dorsal or ventral midlines in the periphery whereas the dendrites of lateral md-das do not approach the dorsal or ventral midline, because of their lateral location, and also do not approach the CNS midline. This suggests that there could be some relationship between proximity to the midline in the periphery and proximity to the midline in the CNS. Those md-das in the dorsal and ventral region that do not project across the midline in the CNS have the most restricted dendritic arborisation patterns in the periphery and do not reach the midline. However the md-da with restricted, dorsally projecting, dendritic arbours that project towards the dorsal midline also crosses the midline in the CNS. This indicates that it is not the dorsal or ventral, as opposed to lateral, location of the md-da cell bodies in the periphery that correlates with the decision to cross the midline in the CNS but the proximity of the dendritic arbours of the md-da neurons to the ventral or dorsal midlines. There therefore does seem to be a somatotopic element to the organisation of the md-da central projections, even though there is no direct correlation between peripheral location and central projection pattern.

6.5.5 The timing of embryonic neurogenesis in relation to the production of sensory neuron clones

Neurogenesis occurs in the peripheral nervous system between 6-9 hrs of embryonic development. The peripheral nervous system is generated in a stereotyped manner with ch and md precursors completing DNA replication earlier than es precursors, and with dorsal

precursors in each class completing their DNA replication earlier than ventral precursors (Bodmer et al., 1989). Almost all md precursors, with the exception of two to three of the ventral mds have completed DNA replication by 7 hrs of embryonic development (Bodmer et al., 1989). As the MARCM technique can only induce loss of the GAL80 repressor in mitotic cells and dorsal mds are frequently labelled, this indicates that when embryos are heatshocked at 3 hrs AEL clones are being induced at, or shortly after the initiation of neurogenesis in the peripheral nervous system. This suggests that the time taken for FLP to be transcribed in response to heatshock, translated, and to catalyse recombination at its FRT target sites is approximately three hours. Labelling of every sensory neuron, with the exception of the vesA and vesB neurons, has been observed when the heatshock stimulus is administered at 3hrs AEL although es neurons have been detected less frequently than other classes of sensory neuron. This was unexpected as es precursors complete their DNA replication slightly later than md and ch precursors, and the vesA and vesB neurons are among the last to be produced. All es neurons would therefore be expected to be labelled as frequently as any other class of neuron. One possible explanation for the lack of vesA and vesB neurons so far observed could be their extremely ventral position in the periphery, which could possibly have lead to their being missed during screening at first, as it was not initially realised quite how ventrally located these neurons are in the larvae compared with the embryonic sensory nervous system. Embryos are also being heatshocked slightly later in development, at 4 hours AEL, in the hope of increasing the proportion of es neuron clones recovered.

6.5.6 Resolving the central projection pattern in the dorso-ventral axis

While examining central projections from both dorsal and ventral angles enables the extent of the axonal processes in the anterior-posterior and medio-lateral axes to be resolved, it is impossible to accurately determine the dorso-ventral extent of these processes from such an angle. While two preparations that had fortuitously twisted onto their side showed that ch neurons - or lch5.3 at least - project to a medio-ventral region of neuropil and that two ddas project to a more dorsal region of neuropil, one of which also produces a comparatively ventral axonal process (Figure 6.7), these preparations also highlight the three-dimensionality of the axonal projections and the extent of the dorso-ventral information that is not being retrieved. Sectioning will be performed to resolve the differences in central projection pattern between neurons in the dorso-ventral plane, once all analysis of the projections in the anterior-posterior and medio-lateral plane is complete.

6.5.7 Future improvements to the technique

The main problem encountered during the characterisation of the central projection patterns of larval sensory neurons has been the frequent simultaneous induction of central and sensory clones. This was expected as the P[GAL4] enhancer-trap line used to provide tissue-specificity was the pan-neuronal elav-GAL4. At the inception of this project no P[GAL4] enhancer-trap lines were available that specifically labelled all sensory neurons and so the elav-GAL4 line provides the best means of creating nervous system-specific mosaics. The difficulties presented by central clones for the interpretation of sensory neuron central projections had not been fully appreciated (particularly as central clones have a tendency to appear in the same segment as the sensory clone of interest) however. The use of more specific GAL4 lines that label sensory neurons or subsets of sensory neurons and most importantly do not label any central neurons would greatly aid the characterisation of the larval pattern of central projections. Several of the lines identified during the previous embryonic enhancer-trap screen (see Chapter 3), which label subsets of sensory neurons but no cells within the CNS, could provide such specificity and may prove extremely useful in future for restricting mosaicism to particular groups of sensory neurons. Additionally, a sensory neuron-specific GAL4 line has now been described (Hummel et al., 2000) which could also be recombined into the MARCM genetic background to restrict mosaicism to the sensory nervous system.

Chapter 7: Conclusions and Future Work

The aim of this work was to identify candidate genes that could specify positional information in sensory neurons and to develop methods of targeting gene expression to single cells in order to analyse whether these candidate genes play a role in specifying central projection pattern. While several of the genes that control what type of sensory neuron is formed have been identified and in some cases their effects on central projection pattern are being uncovered, little is known about how positional values are established in the sensory nervous system. Positional information is provided during early embryonic development by the segmentation genes. Many of genes are known to be required for multiple developmental processes in addition to their role in segmentation, including the specification of cell fate in the embryonic central nervous system. Could segmentation genes also be providing positional information in the sensory nervous system?

7.1 Synopsis of results and main conclusions

A screen to determine the expression patterns of the segmentation gene products revealed that very few of these genes are re-expressed in the embryonic sensory nervous system at any stage during its development (Chapter 2). Only 3 segmentation genes are expressed in subsets of sensory neurons: *engrailed*, *armadillo* and *runt*. Of these, the expression of *armadillo* in all mechanosensory neurons and the localisation of Armadillo staining to dendrites is more likely to reflect a role in junction formation than an instructive role in sensory neuron development. As segmentation genes are expressed in so few sensory neurons and, in the case of *engrailed*, this expression does not correspond with peripheral position, segmentation genes do not appear to be directly providing positional information within sensory neurons. Rather than segmentation genes being re-expressed during sensory nervous system development, the SOPs may retain positional information from the initial process of patterning the embryo that was present in the epidermis prior to their delamination. Nevertheless, as *engrailed* and *runt* both encode transcription factors they both have the potential to direct neuronal differentiation and are good candidates for specifying cell fate in the sensory nervous system.

Both *armadillo* and *engrailed* were misexpressed in all sensory neurons using the GAL4 enhancer-trap technique (Brand and Perrimon, 1993) to determine whether these genes can influence nervous system development (Chapter 4). This experiment has not yet been performed for *runt* as a UAS-*runt* construct, which would enable *runt* to be expressed in all

neurons, is not publicly available. The phenotype resulting from ectopic expression of *engrailed* caused both the production of additional ectopic neurons, and a loss of chordotonal neurons that cannot easily be explained. While such a crude experiment tells little about *engrailed's* normal role in sensory neuron development, it serves to demonstrate that *engrailed* can influence sensory nervous system development, providing strong support for the idea that *engrailed* may control aspects of sensory neuron development.

To examine the effects of ectopic expression of candidate genes on sensory neuron development in more detail a screen to identify more specific P[GAL4] enhancer-trap lines was also performed. Many of the lines identified are not expressed until after the sensory nervous system is fully formed however, limiting their utility as misexpression vectors. At the same time, techniques that would enable genes of interest to be misexpressed in single identified neurons were developed. Two different approaches were investigated: Laser activated gene expression (Halfon et al., 1997) and MARCM (Lee and Luo, 1999). The idea of using a laser to activate gene expression in single identified neurons is particularly appealing as one would have the ability to select the exact sensory neuron in which gene expression is to be activated. However, while a significant amount of effort was spent on developing a means of constituitively activating gene expression in single cells, the rate at which successful gene activation was achieved was so low - less than 10% of neurons targeted resulted in expression - as to make laser activated gene expression impractical. The MARCM technique also enables gene expression to be targeted to single sensory neurons, through tissue-specific mosaic analysis. Large numbers of mosaic individuals can be generated at a time. In addition, MARCM can be used to perform both loss of function and gain of function analysis of genes of interest. The disadvantage of the MARCM technique, compared to laser gene activation, is that there is no means of controlling exactly where recombination will occur. However, as MARCM is based around the GAL4 enhancer-trap technique, mosaicism can be restricted to smaller subsets on neurons by using more specific GAL4 drivers and it is likely that some of the enhancer-trap lines recovered during the embryonic screen will prove extremely suitable for this purpose.

Before the effects of a given gene on the axonal morphology of a particular sensory neuron can be determined, the normal pattern of central projections for each embryonic sensory neuron needs to be established. By characterising sensory neuron central projection patterns in larvae, where the CNS is many times larger than in the embryo, a greater degree of resolution of the differences between individual neurons can be achieved. While this analysis is not yet complete, preliminary findings show that the axonal projections of different groups

of sensory neurons show specific features and occupy characteristic positions within the neuromere, and that sensory neurons can also be individually identified by their projection pattern. A distinct somatotopic trend was found in the projections of ch neurons, where a shift in peripheral location from ventral to medial is accompanied by a corresponding shift in projection location from lateral to medial in the CNS. This trend may not have been detected in the embryonic CNS (Merritt and Whitington, 1995), due to its small size. An interesting by-product of this analysis was the discovery that different md-das produce distinct patterns of dendritic arbours, and that these differences in dendritic morphology in the periphery are also reflected in the axonal morphology of these neurons within the CNS, suggesting that md-das may be subdivided into different functional groups. There is also some degree of a somatotopic relationship between the proximity of the peripheral dendritic arbours of the md-das to either the dorsal or ventral midline and whether an md-da produces a contralateral process in the CNS.

There is also potentially some correlation between sensory projection pattern and segmentation gene expression. The dorsal cluster neuron that expresses Engrailed appears to be the md-td dorsal cluster neuron when observed in 3rd instar larvae using the elav-GAL4 enhancer-trap line. Expression of Engrailed would therefore be limited to tracheal dendrite neurons in the fully formed embryonic sensory nervous system. No clear examples of the axonal morphology of the Engrailed-expressing lateral tracheal dendrite neuron, ltd, have yet been recovered however to determine whether the engrailed-expressing neurons project to a distinct region of the neuropile. Whether Runt, which is expressed in a number of ventral cluster md-das, correlates with the different peripheral dendritic arborisation patterns and central projection patterns of the md-das will also need to be determined.

7.2 Future Work

Once the characterisation of the central projections of embryonic sensory neurons in the larval CNS is completed, the effects of ectopic expression and loss of function mutations in candidate genes on axonal projections can begin to be examined. Both *engrailed* and *runt* are strong candidates for specifying aspects of cell fate in the sensory nervous system but as *engrailed* has already been shown to determine axon morphology in the cockroach (Marie et al., 2000), *engrailed* will be examined first. To examine the native function of *engrailed* in sensory neurons, different mutant alleles of *engrailed* can be recombined into the MARCM background and clones of the sensory neurons that normally express *engrailed* can be generated that are homozygous for *engrailed* loss of function mutations. The MARCM

technique can also be used to induce ectopic expression of *engrailed* in individual sensory neurons, by recombining the UAS-*engrailed* construct into the MARCM set. An examination of the sensory neuron central projections will show whether there is a change in axonal morphology in the absence of Engrailed function or when Engrailed is ectopically expressed.

In addition, as the Engrailed-expressing sensory neurons persist through metamorphosis, raising the possibility that they persist because they express *engrailed*, it would be interesting to examine the effects of *engrailed* loss of function and ectopic expression on the fate of sensory neurons. If significant changes in axon morphology are produced as a result of *engrailed* loss of function, and as neurons that include the Engrailed-expressing neurons guide the growth of adult sensory axons in the CNS, it would be interesting to also examine the effects of *engrailed* loss of function on the central projections of adult sensory neurons. The effects of *engrailed* overexpression in the neurons that normally express Engrailed can also be examined, both using the engrailed-GAL4 line to drive UAS-*engrailed* in all *engrailed*-expressing neurons, and by recombining the engrailed-GAL4 line into the MARCM set to examine the effects of *engrailed* overexpression in individual *engrailed*-expressing neurons.

Although *engrailed* has been shown to negatively regulate *connectin* and *neuroglian* in the central nervous system (Siegler and Jia, 1999), no obvious downstream targets of *engrailed* were identified among the genes tested so far. The monoclonal antibody Mab4C94, which recognises an unidentified cell surface antigen is expressed in four of the five lch5 neurons and could therefore potentially be regulated by *engrailed* (Kolodziej et al., 1995). As this antiserum has just been obtained, this possibility can now be tested. It would also be possible to explore gene array approaches to search for genes that could potentially be regulated by engrailed in sensory neurons (White et al., 1999).

The characterisation of the central projections of sensory neurons in the larval CNS also provides a framework in which the effects of electrical activity and competition in refining sensory neuron central projections could be examined. It has previously been shown that, while the anatomical segregation of sensory afferents clearly influences synaptic connectivity, competition shapes the final pattern of synaptic connectivity and regulates the efficacy of synaptic contacts (Shepherd and Murphey, 1986) (Bacon and Blagburn, 1992). The effects of ablating individual sensory neurons on the central projections formed by the remaining neurons could be examined. For example, different lch5 neurons could be ablated using a laser and the effect of this ablation on the axonal morphology of the remaining sensory

neurons could be determined. Individual neurons could be visualised using dye-injections or by recombining one of the GAL4 lines that specifically labels the lch5 chordotonal neurons into the MARCM set. This would initially establish that competition also regulates the final pattern of central projections among larval sensory neurons. The role of electrical activity in refining the final pattern of central projections would be extremely interesting to explore and it could be determined whether competition between sensory neurons is mediated by electrical activity. A modified version of the Shaker channel has been generated which can suppress electrical activity (White et al., 2001), and this UAS-EKO construct could be used to examine the effects of electrically silencing a sensory neuron on the final pattern of central projections and on the projections of surrounding neurons.

Appendix A

Drosophila Saline

0.15g KCL
7.48g NaCl
0.81g $MgCl_2$
0.26g $CaCl_2$
12.3g Sucrose

pH to 7.4 with NaOH

4% Paraformaldehye

2.26% Na_2HPO_4
2.52% NaOH
4% Paraformaldehyde

X-gal staining solution

31mM $K_3Fe(CN)_5$
31mM $K_4Fe(CN)_6$
0.1M NaH_2PO4
0.1M Na_2HPO_4
1.0M NaCl
0.5M $MgCl_2$

Drosophila Media

5g Agar
35g Flour
75g Sugar
15g Yeast
850ml Distilled water
5% Nipagen

Apple Juice Agar Plates

3g Agar
100ml Water
3g Sugar
33ml Apple juice

Appendix B

Antibody	Host	Dilution	Supplier
22c10	mouse	1:250	DSHB, University of Iowa
armadillo	mouse	1:40	DSHB, University of Iowa
atonal	rabbit	1:5000	A. Jarman, University of Edinburgh
barH1	rabbit	1:40	T. Kojima, University of Tokyo
bicoid	rat	1:200	M. Ruiz-Gomez, University of Cambridge
caudal	guinea pig	1:1000	M. Ruiz-Gomez, University of Cambridge
chaoptin	mouse	1:200	DSHB, University of Iowa
connectin	mouse	1:5	R. White, University of Cambridge
cubitus interrupt	rat	1:4	R. Holmgren, Northwestern University
cut	mouse	1:20	DSHB, University of Iowa
dishevilled	rat	1:200/1:100	S. Pronovost, Stanford University
elav	mouse	1:1000	DSHB, University of Iowa
engrailed	mouse	1:3	DSHB, University of Iowa
even-skipped	mouse	1:200	M. Ruiz-Gomez, University of Cambridge
fasciclin I	mouse	1:200/1:100	DSHB, University of Iowa
fasciclin II	mouse	1:200	DSHB, University of Iowa
fasciclin III	mouse	1:500	DSHB, University of Iowa
fushi-tarazu	rat	1:200	M. Ruiz-Gomez, University of Cambridge
giant	rabbit	1:1000	M. Ruiz-Gomez, University of Cambridge
glass	mouse	1:200	DSHB, University of Iowa
gooseberry-p	rat	1:4	R. Holmgren, Northwestern University
gooseberry-d	rat	1:4	R. Holmgren, Northwestern University
hairy	rat	1:200	M. Ruiz-Gomez, University of Cambridge
hedgehog	rat	1:200/1:100	I, Guerrero
hindsight	mouse	1:20	DSHB, University of Iowa
hunchback	rat	1:1000	M. Ruiz-Gomez, University of Cambridge
ILMP-2	rat	1:200	J. Natzle,University of California, Davis
islet	mouse	1:200	DSHB, University of Iowa
knirps	guinea pig	1:200	M. Ruiz-Gomez, University of Cambridge
kruppel	rabbit	1:1500	C. Rushlow, NYU
neuroglian	mouse	1:200	DSHB, University of Iowa
neurotactin	mouse	1:200	DSHB, University of Iowa
odd-skipped	guinea pig	1:200	M. Ruiz-Gomez, University of Cambridge
paired	guinea pig	1:1000	M. Ruiz-Gomez, University of Cambridge
patched	mouse	1:100	M. Ruiz-Gomez, University of Cambridge
peanut	mouse	1:04	DSHB, University of Iowa
pox-n	rabbit	1:40	W. Boll, University of Zurich
rough	mouse	1:200/1:100	DSHB, University of Iowa
runt	guinea pig	1:1000	M. Ruiz-Gomez, University of Cambridge
scabrous	mouse	1:200	DSHB, University of Iowa
slit	mouse	1:200	DSHB, University of Iowa
sloppy-paired	guinea pig	1:200	M. Ruiz-Gomez, University of Cambridge
tailless	rabbit	1:200	M. Ruiz-Gomez, University of Cambridge
wingless	mouse	1:100	R. Nusse

Bibliography

Ahmad, K., and Henikoff, S. (2001). Modulation of a transcription factor counteracts heterochromatic gene silencing in Drosophila. Cell *104*, 839-847.

Alonso, L. A. G., and Garcia-Bellido, A. (1988). Extramacrochaetae, a Trans-Acting Gene Of the Achaete-Scute Complex Of Drosophila Involved In Cell Communication. Rouxs Archives Of Developmental Biology *197*, 328-338.

Alonso, M. C., and Cabrera, C. V. (1988). The Achaete-Scute Gene-Complex Of Drosophila-Melanogaster Comprises 4 Homologous Genes. Embo Journal *7*, 2585-2591.

Altner, H., Routil, C., and Loftus, R. (1981). The Structure of Bimodal Chemoreceptive, Thermoreceptive, and Hygroreceptive Sensilla on the Antenna of Locusta-Migratoria. Cell and Tissue Research *215*, 289-308.

Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M. E. (1995). Notch Signaling. Science *268*, 225-232.

Artavanis-Tsakonas, S., and Simpson, P. (1991). Choosing a Cell Fate - a View from the Notch Locus. Trends in Genetics *7*, 403-408.

Atkins, G., and Pollack, G. S. (1987). Correlations between structure, topographic arrangement, and spectral sensitivity of sound-sensitive interneurons in crickets. Journal of Comparative Neurology *266*, 398-412.

Awasaki, T., Saito, M., Sone, M., Suzuki, E., Sakai, R., Ito, K., and Hama, C. (2000). The Drosophila trio plays an essential role in patterning of axons by regulating their directional extension. Neuron *26*, 119-131.

Bacon, J. P., and Blagburn, J. M. (1992). Ectopic Sensory Neurons In Mutant Cockroaches Compete With Normal- Cells For Central Targets. Development *115*, 773-784.

Bacon, J. P., and Murphey, R. K. (1984). Receptive-Fields Of Cricket Giant Interneurones Are Related to Their Dendritic Structure. Journal Of Physiology-London *352*, 601-623.

Bashaw, G. J., and Goodman, C. S. (1999). Chimeric axon guidance receptors: The cytoplasmic domains of slit and netrin receptors specify attraction versus repulsion. Cell *97*, 917-926.

Bastiani, M. J., Harrelson, A. L., Snow, P. M., and Goodman, C. S. (1987). Expression Of Fasciclin-I and Fasciclin-Ii Glycoproteins On Subsets Of Axon Pathways During Neuronal Development In the Grasshopper. Cell *48*, 745-755.

Bastiani, M. J., Raper, J. A., and Goodman, C. S. (1984). Pathfinding By Neuronal Growth Cones In Grasshopper Embryos .3. Selective Affinity Of the G-Growth Cone For the P-Cells Within the a/P Fascicle. Journal Of Neuroscience *4*, 2311-2328.

Bate, C. (1976). Pioneer neurons in an embryo. Nature(Lond) *260*.

Bateman, J., Shu, H., and Van Vactor, D. (2000). The guanine nucleotide exchange factor trio mediates axonal development in the Drosophila embryo. Neuron *26*, 93-106.

Baumgartner, S., Martin, D., Hagios, C., and Chiquetehrismann, R. (1994). Ten(M), a Drosophila Gene-Related to Tenascin, Is a New Pair- Rule Gene. Embo Journal *13*, 3728-3740.

Bentley, D., and Caudy, M. (1983). Pioneer Axons Lose Directed Growth After Selective Killing Of Guidepost Cells. Nature *304*, 62-65.

Bentley, D., and Keshishian, H. (1982). Pathfinding By Peripheral Pioneer Neurons In Grasshoppers. Science *218*, 1082-1088.

Bentley, D., and O'Connor, T. P. (1994). Cytoskeletal events in growth cone steering. Current Opinion in Neurobiology *4*, 43-48.

Bier, E., Vaessin, H., Shepherd, S., Lee, K., McCall, K., Barbel, S., Ackerman, L., Carretto, R., Uemura, T., Grell, E., *et al.* (1989). Searching For Pattern and Mutation In the Drosophila Genome With a P- Lacz Vector. Genes & Development *3*, 1273-1287.

Blagburn, J. M., Gibbon, C. R., and Bacon, J. P. (1995). Expression of engrailed in an array of identified sensory neurons - comparison with position, axonal arborization, and synaptic connectivity. Journal of Neurobiology *28*, 493-505.

Blochlinger, K., Bodmer, R., Jan, L. Y., and Jan, Y. N. (1990). Patterns Of Expression Of Cut, a Protein Required For External Sensory Organ Development In Wild-Type and Cut Mutant Drosophila Embryos. Genes & Development *4*, 1322-1331.

Blochlinger, K., Jan, L. Y., and Jan, Y. N. (1991). Transformation of sensory organ identity by ectopic expression of Cut in Drosophila. Genes & Development *5*, 1124-1135.

Bodmer, R., Carretto, R., and Jan, Y. N. (1989). Neurogenesis of the Peripheral Nervous-System in Drosophila Embryos - DNA-Replication Patterns and Cell Lineages. Neuron *3*, 21-32.

Braitenburg, V. (1972). Periodic structures and structural gradients in the visual ganglia of the fly. In Information processing in the visual systems of arthropods. R. Wehner., ed. (Springer, Berlin) 3-15.

Brand, A. H., and Dormand, E. L. (1995). The Gal4 System As a Tool For Unraveling the Mysteries Of the Drosophila Nervous-System. Current Opinion In Neurobiology *5*, 572-578.

Brand, A. H., and Perrimon, N. (1993). Targeted Gene-Expression As a Means Of Altering Cell Fates and Generating Dominant Phenotypes. Development *118*, 401-415.

Breidbach, O. (1990). Metamorphic changes in the central projections of hair sensilla in Tenebrio molitor L. (Insecta:Coleoptera). Cell Tissue Res *259*, 159-176.

Breiling, A., Turner, B. M., Bianchi, M. E., and Orlando, V. (2001). General transcription factors bind promoters repressed by Polycomb group proteins. Nature *412*, 651-655.

Brewster, R., and Bodmer, R. (1995). Origin and Specification Of Type-II Sensory Neurons In Drosophila. Development *121*, 2923-2936.

Broadus, J., Skeath, J. B., Spana, E. P., Bossing, T., Technau, G., and Doe, C. Q. (1995). New neuroblast markers and the origin of the Acc/Pcc neurons in the Drosophila Central-Nervous-System. Mechanisms of Development *53*, 393-402.

Burrows, M., and Siegler, M. V. S. (1984). The Morphological Diversity and Receptive-Fields Of Spiking Local Interneurons In the Locust Metathoracic Ganglion. Journal Of Comparative Neurology *224*, 483-508.

Cabrera, C. V., and Alonso, M. C. (1991). Transcriptional Activation By Heterodimers Of the Achaete Scute and Daughterless Gene-Products Of Drosophila. Embo Journal *10*, 2965-2973.

Cabrera, C. V., Alonso, M. C., and Huikeshoven, H. (1994). Regulation Of Scute Function By Extramacrochaete In-Vitro and In-Vivo. Development *120*, 3595-3603.

Cabrera, C. V., Martinez-Arias, A., and Bate, M. (1987). The Expression Of 3 Members Of the Achaete-Scute Gene-Complex Correlates With Neuroblast Segregation In Drosophila. Cell *50*, 425-433.

Callahan, C. A., Muralidhar, M. G., Lundgren, S. E., Scully, A. L., and Thomas, J. B. (1995). Control of neuronal pathway selection by a Drosophila receptor protein-tyrosine kinase family member. Nature *376*, 171-174.

Campuzano, S., and Modolell, J. (1992). Patterning Of the Drosophila Nervous-System - the Achaete-Scute Gene- Complex. Trends In Genetics *8*, 202-208.

Chiba, A., Hing, H., Cash, S., and Keshishian, H. (1993). Growth Cone Choices Of Drosophila Motoneurons In Response to Muscle- Fiber Mismatch. Journal Of Neuroscience *13*, 714-732.

Chou, T. B., and Perrimon, N. (1992). Use of a Yeast Site-Specific Recombinase to Produce Female Germline Chimeras in Drosophila. Genetics *131*, 643-653.

Coulter, D., and Wieschaus, E. (1986). Segmentation Genes and the Distributions of Transcripts. Nature *321*, 472-474.

Cox, R. T., Kirkpatrick, C., and Peifer, M. (1996). Armadillo is required for adherens junction assembly, cell polarity, and morphogenesis during Drosophila embryogenesis. Journal of Cell Biology *134*, 133-148.

Cubas, P., Decelis, J. F., Campuzano, S., and Modolell, J. (1991). Proneural Clusters Of Achaete-Scute Expression and the Generation Of Sensory Organs In the Drosophila Imaginal Wing Disk. Genes & Development *5*, 996-1008.

Cubas, P., and Modolell, J. (1992). The Extramacrochaetae Gene Provides Information for Sensory Organ Patterning. Embo Journal *11*, 3385-3393.

Culi, J., and Modolell, J. (1998). Proneural gene self-stimulation in neural precursors: an essential mechanism for sense organ development that is regulated by Notch signaling. Genes & Development *12*, 2036-2047.

Dambly-Chaudiere, C., Jamet, E., Burri, M., Bopp, D., Basler, K., Hafen, E., Dumont, N., Spielmann, P., Ghysen, A., and Noll, M. (1992). The paired box gene pox-neuro - a determinant of poly- innervated sense-organs in Drosophila. Cell *69*, 159-172.

Dambly-Chaudiere, C., and Ghysen, A. (1986). The Sense-Organs In the Drosophila Larva and Their Relation to the Embryonic Pattern Of Sensory Neurons. Rouxs Archives Of Developmental Biology *195*, 222-228.

Dambly-Chaudiere, C., and Ghysen, A. (1987). The Pattern Of Sense-Organs In Normal and Scute Embryos. Journal Of Neurogenetics *4*, 122-122.

Dang, D. T., and Perrimon, N. (1992). Use of a Yeast Site-Specific Recombinase to Generate Embryonic Mosaics in Drosophila. Developmental Genetics *13*, 367-375.

Del Prado, J. M., and Garcia-Bellido, A. (1984). Genetic-Regulation Of the Achaete-Scute Complex Of Drosophila- Melanogaster. Wilhelm Rouxs Archives Of Developmental Biology *193*, 242-245.

de la Escalera, S., Martin, M. D., and Jiminez, F. (1991). Characterisation and Gene Cloning of Neurotactin, a Drosophila transmembrane protein related to cholinesterases. EMBO Journal *9*, 3593-3601.

Desai, C. J., Gindhart, J. G., Goldstein, L. S. B., and Zinn, K. (1996). Receptor tyrosine phosphatases are required for motor axon guidance in the Drosophila embryo. Cell *84*, 599-609.

Dickson, B. J. (2001). Rho GTPases in growth cone guidance. Current Opinion in Neurobiology *11*, 103-110.

Dinardo, S., Sher, E., Heemskerkjongens, J., Kassis, J. A., and Ofarrell, P. H. (1988). 2-Tiered Regulation of Spatially Patterned Engrailed Gene- Expression During Drosophila Embryogenesis. Nature *332*, 604-609.

Doe, C. Q. (1992). Molecular Markers For Identified Neuroblasts and Ganglion Mother Cells In the Drosophila Central-Nervous-System. Development *116*, 855.

Dominguez, M., and Campuzano, S. (1993). Asense, a Member Of the Drosophila-Achaete Scute Complex, Is a Proneural and Neural Differentiation Gene. Embo Journal *12*, 2049-2060.

Elkins, T., Zinn, K., McAllister, L., Hoffmann, F. M., and Goodman, C. S. (1990). Genetic-Analysis Of a Drosophila Neural Cell-Adhesion Molecule - Interaction Of Fasciclin-I and Abelson Tyrosine Kinase Mutations. Cell *60*, 565-575.

Ellis, H. M., Spann, D. R., and Posakony, J. W. (1990). Extramacrochaetae, a Negative Regulator of Sensory Organ Development in Drosophila, Defines a New Class of Helix-Loop- Helix Proteins. Cell *61*, 27-38.

Epps, J. L., Jones, J. B., and Tanda, S. (1997). oroshigane, a new segment polarity gene of Drosophila melanogaster, functions in hedgehog signal transduction. Genetics *145*, 1041-1052.

Fambrough, D., and Goodman, C. S. (1996). The Drosophila beaten path gene encodes a novel secreted protein that regulates defasciculation at motor axon choice points. Cell *87*, 1049-1058.

Fan, J. H., Mansfield, S. G., Redmond, T., Gordonweeks, P. R., and Raper, J. A. (1993). The Organization of F-Actin and Microtubules in Growth Cones Exposed to a Brain-Derived Collapsing Factor. Journal of Cell Biology *121*, 867-878.

Ferveur, J. F., Savarit, F., O'Kane, C. J., Sureau, G., Greenspan, R. J., and Jallon, J. M. (1997). Genetic feminization of pheromones and its behavioral consequences in Drosophila males. Science *276*, 1555-1558.

Fjose, A., McGinnis, W. J., and Gehring, W. J. (1985). Isolation of a Homeo Box-Containing Gene from the Engrailed Region of Drosophila and the Spatial-Distribution of Its Transcripts. Nature *313*, 284-289.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. Cell *87*, 651-660.

French, V. I., Bryant, P. J., and Bryant, S. V. (1976). Pattern regulation in epimorphic fields. Science *193*, 969-981.

Friedman, G. C., and Oleary, D. D. M. (1996). Retroviral misexpression of engrailed genes in the chick optic tectum perturbs the topographic targeting of retinal axons. Journal of Neuroscience *16*, 5498-5509.

Fujita, S. C., Zipursky, S. L., Benzer, S., Ferrus, A., and Shotwell, S. L. (1982). Monoclonal-Antibodies against the Drosophila Nervous-System. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences *79*, 7929-7933.

Garbe, J. C., Yang, E., and Fristrom, J. W. (1993). Imp-L2 - an Essential Secreted Immunoglobulin Family Member Implicated In Neural and Ectodermal Development In Drosophila. Development *119*, 1237-1250.

Garcia-Bellido, A. (1979). Genetic analysis of the achaete-scute system of Drosophila melanogaster. Genetics *91*, 491-520.

Garrell, J., and Modolell, J. (1990). The Drosophila Extramacrochaetae Locus, an Antagonist of Proneural Genes That, Like These Genes, Encodes a Helix-Loop- Helix Protein. Cell *61*, 39-48.

Ghysen, A. (1980). The Projection of Sensory Neurons in the Central Nervous System of Drosophila: Choice of the Appropriate Pathway. Developmental Biology *78*, 521 -541.

Ghysen, A., Dambly-Chaudiere, C., Aceves, E., Jan, L. Y., and Jan, Y. N. (1986). Sensory neurons and peripheral pathways in Drosophila embryos. Rouxs Archives of Developmental Biology *195*, 281-289.

Ghysen, A., and O'Kane, C. (1989). Neural Enhancer-Like Elements As Specific Cell Markers In Drosophila. Development *105*, 35-52.

Ghysen, A., and Dambly-Chaudiere, C. (1993). The Specification Of Sensory Neuron Identity In Drosophila. Bioessays *15*, 293-298.

Glossop, N. R. J. (1997). The Developmental Assembly of Sensory Axon Arrays in the Central Nervous System of Drosophila Melanogaster: a combined Cellular, Genetic and Molecular Study. PhD thesis. University of Southampton.

Golic, K. G., and Golic, M. M. (1996). Engineering the Drosophila genome: Chromosome rearrangements by design. Genetics *144*, 1693-1711.

Golic, K. G. (1991). Site-Specific Recombination between Homologous Chromosomes in Drosophila. Science *252*, 958-961.

Golic, K. G., and Lindquist, S. (1989). The Flp Recombinase of Yeast Catalyzes Site-Specific Recombination in the Drosophila Genome. Cell *59*, 499-509.

Golic, M. M., Rong, Y. S., Petersen, R. B., Lindquist, S. L., and Golic, K. G. (1997). FLP-mediated DNA mobilization to specific target sites in Drosophila chromosomes. Nucleic Acids Research *25*, 3665-3671.

Gomez-Skarmeta, J. L., Delcorral, R. D., Delacallemustienes, E., Ferresmarco, D., and Modolell, J. (1996). Araucan and Caupolican, 2 Members Of the Novel Iroquois Complex, Encode Homeoproteins That Control Proneural and Vein-Forming Genes. Cell *85*, 95-105.

Gomez-Skarmeta, J. L., and Modolell, J. (1996). Araucan and Caupolican Provide a Link Between Compartment Subdivisions and Patterning Of Sensory Organs and Veins In the Drosophila Wing. Genes & Development *10*, 2935-2945.

Goodman, C. S. (1984). Landmarks and Labels That Help Developing Neurons Find Their Way. Bioscience *34*, 300-307.

Goodman, C. S., Bastiani, M. J., Doe, C. Q., Dulac, S., Helfand, S. L., Kuwada, J. Y., and Thomas, J. B. (1984). Cell Recognition During Neuronal Development. Science *225*, 1271-1279.

Goodman, C. S., and Bate, M. (1981). Neuronal Development In the Grasshopper. Trends In Neurosciences *4*, 163-169.

Goodman, C. S., Bate, M., and Spitzer, N. C. (1981). Embryonic development of identified neurons: Origin and transformation of the H Cell. The Journal Of Neuroscience *1*, 94-102.

Goodman, C. S., Raper, J. A., Chang, S., and Ho, R. (1983). Grasshopper Growth Cones - Divergent Choices and Labeled Pathways. Progress In Brain Research *58*, 283-304.

Goulding, S. E., zur Lage, P., and Jarman, A. P. (2000). amos, a proneural gene for Drosophila olfactory sense organs that is regulated by lozenge. Neuron *25*, 69-78.

Grenningloh, G., Rehm, E. J., and Goodman, C. S. (1991). Genetic-Analysis Of Growth Cone Guidance In Drosophila - Fasciclin-II Functions As a Neuronal Recognition Molecule. Cell *67*, 45-57.

Grillenzoni, N., vanHelden, J., Dambly-Chaudiere, C., and Ghysen, A. (1998). The iroquois complex controls the somatotopy of Drosophila notum mechanosensory projections. Development *125*, 3563-3569.

Grueber, W. B., and Truman, J. W. (1999). Development and organization of a nitric-oxide-sensitive peripheral neural plexus in larvae of the moth, Manduca sexta. Journal Of Comparative Neurology *404*, 127-141.

Gustafson, K., and Boulianne, G. L. (1996). Distinct expression patterns detected within individual tissues by the GAL4 enhancer trap technique. Genome *39*, 174-182.

Halder, G., Callaerts, P., and Gehring, W. J. (1995). Induction of Ectopic Eyes by Targeted Expression of the Eyeless Gene in Drosophila. Science *267*, 1788-1792.

Halfon, M. S., Kose, H., Chiba, A., and Keshishian, H. (1997). Targeted gene expression without a tissue-specific promoter: Creating mosaic embryos using laser-induced single-cell heat shock. Proceedings Of the National Academy Of Sciences Of the United States Of America *94*, 6255-6260.

Hall, A. (1994). Small Gtp-Binding Proteins and the Regulation of the Actin Cytoskeleton. Annual Review of Cell Biology *10*, 31-54.

Harrelson, A. L., and Goodman, C. S. (1988). Growth Cone Guidance In Insects - Fasciclin-Ii Is a Member Of the Immunoglobulin Superfamily. Science *242*, 700-708.

Harrison, D. A., and Perrimon, N. (1993). Simple and Efficient Generation of Marked Clones in Drosophila. Current Biology *3*, 424-433.

Hartenstein, V. (1988). Development of Drosophila larval sensory organs - Spatiotemporal pattern of sensory neurons, peripheral axonal pathways and sensilla differentiation. Development *102*, 869-886.

Hassan, B., and Vaessin, H. (1997). Daughterless is required for the expression of cell cycle genes in peripheral nervous system precursors of Drosophila embryos. Developmental Genetics *21*, 117-122.

Heuer, J. G., and Kaufman, T. C. (1992). Homeotic Genes Have Specific Functional Roles in the Establishment of the Drosophila Embryonic Peripheral Nervous- System. Development *115*, 35-47.

Hidalgo, A., Urban, J., and Brand, A. H. (1995). Targeted ablation of glia disrupts axon tract formation in the Drosophila CNS. Development *121*, 3703-3712.

Higashijima, S., Michiue, T., Emori, Y., and Saigo, K. (1992). Subtype Determination Of Drosophila Embryonic External Sensory Organs By Redundant Homeo Box Genes Barh1 and Barh2. Genes & Development *6*, 1005-1018.

Hing, H., Xiao, J., Harden, N., Lim, L., and Zipursky, S. L. (1999). Pak functions downstream of dock to regulate photoreceptor axon guidance in Drosophila. Cell *97*, 853-863.

Hinz, U., Giebel, B., and Campos-Ortega, J. A. (1994). The Basic-Helix-Loop-Helix Domain of Drosophila Lethal of Scute Protein Is Sufficient for Proneural Function and Activates Neurogenic Genes. Cell *76*, 77-87.

Ho, R. K., and Goodman, C. S. (1982). Peripheral Pathways Are Pioneered By an Array Of Central and Peripheral Neurons In Grasshopper Embryos. Nature *297*, 404-406.

Hoch, M., and Jäckle, H. (1993). Transcriptional regulation and spatial patterning in Drosophila. Current Opinion in Genetics and Development *3*, 566-573.

Horridge, G. A., and Meinertshagen, I. A. (1970). The accuracy of the patterns of connexions of the first- and second-order neurons of the visual system of Calliphora. Proceedings of the Royal Society London B. *175*, 69-82.

Howard, K., and Ingham, P. (1986). Regulatory Interactions between the Segmentation Genes Fushi- Tarazu, Hairy, and Engrailed in the Drosophila Blastoderm. Cell *44*, 949-957.

Hu, S., Sonnenfeld, M., Stahl, S., and Crews, S. T. (1998). Midline fasciclin: A Drosophila Fasciclin-I-related membrane protein localized to the CNS midline cells and trachea. Journal Of Neurobiology *35*, 77-93.

Huang, A. M., Rusch, J., and Levine, M. (1997). An anteroposterior dorsal gradient in the Drosophila embryo. Genes & Development *11*, 1963-1973.

Huang, M. L., Hsu, C. H., and Chien, C. T. (2000). The proneural gene amos promotes multiple dendritic neuron formation in the Drosophila peripheral nervous system. Neuron *25*, 57-67.

Hummel, T., Krukkert, K., Roos, J., Davis, G., and Klambt, C. (2000). Drosophila Futsch/22C10 is a MAP1B-like protein required for dendritic and axonal development. Neuron *26*, 357-370.

Hunter, C., and Wieschaus, E. (2000). Regulated expression of nullo is required for the formation of distinct apical and basal adherens junctions in the Drosophila blastoderm. Journal of Cell Biology *150*, 391-401.

Husterert, R. (1978). Segmental and interganglionic projections from primary fibres of insect mechanoreceptors. Cell Tissue Research. *194*, 337-351.

Ingham, P., Martinez -Arias, A., Lawrence, P. A., and Howard, K. (1985a). Expression of Engrailed in the Parasegment of Drosophila. Nature *317*, 634-636.

Ingham, P. W., Pinchin, S. M., Howard, K. R., and Ish-Horowicz, D. (1985b). Genetic-Analysis of the Hairy Locus in Drosophila-Melanogaster. Genetics *111*, 463-486.

Isbister, C. M., Tsai, A., Wong, S. T., Kolodkin, A. L., and O'Connor, T. P. (1999). Discrete roles for secreted and transmembrane semaphorins in neuronal growth cone guidance in vivo. Development *126*, 2007-2019.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y., and Yamamoto, D. (1997). The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. Development *124*, 761-771.

Jacobs, G. A., and Miller, J. P. (1985). Functional Properties of Individual Neuronal Branches Isolated in Situ by Laser Photoinactivation. Science *228*, 344-346.

Jacobs, J. R. (1993). Perturbed Glial Scaffold Formation Precedes Axon Tract Malformation In Drosophila Mutants. Journal Of Neurobiology *24*, 611-626.

Jacobs, J. R., and Goodman, C. S. (1989a). Embryonic-Development Of Axon Pathways In the Drosophila Cns .1. a Glial Scaffold Appears Before the 1st Growth Cones. Journal Of Neuroscience *9*, 2402-2411.

Jacobs, J. R., and Goodman, C. S. (1989b). Embryonic-Development Of Axon Pathways In the Drosophila Cns .2. Behavior Of Pioneer Growth Cones. Journal Of Neuroscience *9*, 2412-2422.

Jarman, A. P., Grau, Y., Jan, L. Y., and Jan, Y. N. (1993). Atonal Is a Proneural Gene That Directs Chordotonal Organ Formation In the Drosophila Peripheral Nervous-System. Cell *73*, 1307-1321.

Jarman, A. P., Sun, Y., Jan, L. Y., and Jan, Y. N. (1995). Role Of the Proneural Gene, Atonal, In Formation Of Drosophila Chordotonal Organs and Photoreceptors. Development *121*, 2019-2030.

Jay, D. G., and Keshishian, H. (1990). Laser Inactivation Of Fasciclin-I Disrupts Axon Adhesion Of Grasshopper Pioneer Neurons. Nature *348*, 548-550.

Jennings, B., Decelis, J., Delidakis, C., Preiss, A., and Bray, S. (1995). Role of Notch and Achaete-Scute Complex in the Expression of Enhancer of Split Bhlh Proteins. Development *121*, 3745-3752.

Jennings, B., Preiss, A., Delidakis, C., and Bray, S. (1994). The Notch Signaling Pathway Is Required for Enhancer of Split Bhlh Protein Expression During Neurogenesis in the Drosophila Embryo. Development *120*, 3537-3548.

Jimenez, F., Martinmorris, L. E., Velasco, L., Chu, H., Sierra, J., Rosen, D. R., and White, K. (1995). Vnd, a Gene Required for Early Neurogenesis of Drosophila, Encodes a Homeodomain Protein. Embo Journal *14*, 3487-3495.

Johnson, S. E., and Murphey, R. K. (1985). The Afferent Projection Of Mesothoracic Bristle Hairs In the Cricket, Acheta-Domesticus. Journal Of Comparative Physiology a-Sensory Neural and Behavioral Physiology *156*, 369-379.

Jones, B. W. (2001). Glial cell development in the Drosophila embryo. Bioessays *23*, 877-887.

Jurgens, G., Wieschaus, E., Nüsslein-Volhard, C., and Kluding, H. (1984). Mutations Affecting the Pattern of the Larval Cuticle in Drosophila-Melanogaster .2. Zygotic Loci on the 3rd Chromosome. Wilhelm Rouxs Archives of Developmental Biology *193*, 283-295.

Kaufmann, N., Wills, Z. P., and VanVactor, D. (1998). Drosophila Rac1 controls motor axon guidance. Development *125*, 453-461.

Keshishian, H. (1980). The Origin and Morphogenesis of Pioneer Neurons in the Grasshopper Metathoracic Leg. Developmental Biology *80*, 388-397.

Keshishian, H., and Bentley, D. (1983a). Embryogenesis of peripheral nerve pathways in grasshopper legs .1. The initial nerve pathway to the CNS. Developmental Biology *96*, 89-102.

Keshishian, H., and Bentley, D. (1983b). Embryogenesis of peripheral nerve pathways in grasshopper legs .3. Development without pioneer neurons. Developmental Biology *96*, 116-124.

Keshishian, H., and Bentley, D. (1983c). Embryogenesis Of Peripheral-Nerve Pathways In Grasshopper Legs .2. the Major Nerve Routes. Developmental Biology *96*, 103-115.

Kidd, T., Bland, K. S., and Goodman, C. S. (1999). Slit is the midline repellent for the robe receptor in Drosophila. Cell *96*, 785-794.

Kidd, T., Brose, K., Mitchell, K. J., Fetter, R. D., Tessier-Lavigne, M., Goodman, C. S., and Tear, G. (1998). Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell *92*, 205-215.

Klambt, C., and Goodman, C. S. (1991). The diversity and pattern of glia during axon pathway formation in the *Drosophila* embryo. Glia *4*, 205-213.

Klambt, C., Knust, E., Tietze, K., and Campos-Ortega, J. A. (1989). Closely Related Transcripts Encoded by the Neurogenic Gene- Complex Enhancer of Split of Drosophila-Melanogaster. Embo Journal *8*, 203-210.

Klose, M., and Bentley, D. (1989). Transient pioneer neurons are essential for formation of an embryonic peripheral nerve. Science *245*, 982-984.

Kolodkin, A. L. (1996). Semaphorins: Mediators of repulsive growth cone guidance. Trends in Cell Biology *6*, 15-22.

Kolodkin, A. L., Matthes, D. J., O'Connor, T. P., Patel, N. H., Admon, A., Bentley, D., and Goodman, C. S. (1992). Fasciclin-Iv - Sequence, Expression, and Function During Growth Cone Guidance In the Grasshopper Embryo. Neuron *9*, 831-845.

Kolodziej, P. A., Timpe, L. C., Mitchell, K. J., Fried, S. R., Goodman, C. S., Jan, L. Y., and Jan, Y. N. (1996). frazzled encodes a Drosophila member of the dcc immunoglobulin subfamily and is required for CNS and motor axon guidance. Cell *87*, 197-204.

Koushika, S. P., Lisbin, M. J., and White, K. (1996). elav, a Drosophila neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. Current Biology *6*, 1634-1641.

Kunes, S., Wilson, C., and Steller, H. (1993). Independent guidance of retinal axons In the developing visual system of Drosophila. Journal of Neuroscience *13*, 752-767.

Lakes-Harlan, R., Pollack, G. S., and Merritt, D. J. (1991). From embryo to adult - Anatomy and development of a leg sensory organ In Phormia regina, Meigen (Insecta, Diptera) .2. Development and persistence of sensory neurons. Journal of Comparative Neurology *308*, 200-208.

Landgraf, M., Bossing, T., Technau, G. M., and Bate, M. (1997). The origin, location, and projections of the embryonic abdominal motorneurons of Drosophila. Journal Of Neuroscience *17*, 9642-9655.

Lee, T., and Luo, L. Q. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451-461.

Levine, R. B., Pak, C., and Linn, D. (1985). The Structure, Function and Metamorphic Reorganization Of Somatotopically Projecting Sensory Neurons In Manduca-Sexta Larvae. Journal Of Comparative Physiology a-Sensory Neural and Behavioral Physiology *157*, 1-13.

Liebl, E. C., Forsthoefel, D. J., Franco, L. S., Sample, S. H., Hess, J. E., Cowger, J. A., Chandler, M. P., Shupert, A. M., and Seeger, M. A. (2000). Dosage-sensitive, reciprocal genetic interactions between the Abl tyrosine kinase and the putative GEF trio reveal trio's role in axon pathfinding. Neuron *26*, 107-118.

Lin, D. M., Fetter, R. D., Kopczynski, C., Grenningloh, G., and Goodman, C. S. (1994). Genetic-analysis of fasciclin-II in Drosophila - defasciculation, refasciculation, and altered fasciculation. Neuron *13*, 1055-1069.

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

Lin, D. M., Auld, V. J., and Goodman, C. S. (1995). Targeted neuronal cell ablation in the Drosophila embryo - pathfinding by follower growth cones in the absence of pioneers. Neuron *14*, 707-715.

Logan, C., Wizenmann, A., Drescher, U., Monschau, B., Bonhoeffer, F., and Lumsden, A. (1996). Rostral optic tectum acquires caudal characteristics following ectopic Engrailed expression. Current Biology *6*, 1006-1014.

Luo, L., Jan, L. Y., and Jan, Y. N. (1997). Rho family small GTP-binding proteins in growth cone signalling. Current Opinion in Neurobiology *7*, 81-86.

Luo, L. Q., Liao, Y. J., Jan, L. Y., and Jan, Y. N. (1994). Distinct morphogenetic functions of similar small GTPases - Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. Genes & Development *8*, 1787-1802.

Luo, Y. L., Raible, D., and Raper, J. A. (1993). Collapsin - a Protein in Brain That Induces the Collapse and Paralysis of Neuronal Growth Cones. Cell *75*, 217-227.

Marie, B., Bacon, J. P., and Blagburn, J. M. (2000). Double-stranded RNA interference shows that Engrailed controls the synaptic specificity of identified sensory neurons. Current Biology *10*, 289-292.

Martinez, C., and Modolell, J. (1991). Cross-Regulatory Interactions Between the Proneural Achaete and Scute Genes Of Drosophila. Science *251*, 1485-1487.

Matheson, T. (1990). Responses and locations of neurones in the locust metathoracic femoral chordotonal organ. Journal Of Comparative Physiology A *166*, 915-927.

Matthes, D. J., Sink, H., Kolodkin, A. L., and Goodman, C. S. (1995). Semaphorin-ii can function as a selective inhibitor of specific synaptic arborizations. Cell *81*, 631-639.

McDonald, J. A., and Doe, C. Q. (1997). Establishing neuroblast-specific gene expression in the Drosophila CNS: Huckebein is activated by Wingless and Hedgehog and repressed by Engrailed and Gooseberry. Development *124*, 1079-1087.

McDonald, J. A., Holbrook, S., Isshiki, T., Weiss, J., Doe, C. Q., and Mellerick, D. M. (1998). Dorsoventral patterning in the Drosophila central nervous system: the vnd homeobox gene specifies ventral column identity. Genes & Development *12*, 3603-3612.

McIver, S. B. (1985). In Comprehensive Insect Physiology Biochemistry and Pharmacology Volume 6, G. A. Kerkut and L. I. Gilbert, ed. (Oxford: Pergamon Press), pp71-132.

Meinertzhagen, I.A., and Hanson, T.E. (1993). In The Development of Drosophila melanogaster, M. Bate and A. Martinez-Ariaz, ed.(Cold Spring Harbor, NY: Cold Spring Harbor University Press), pp. 1363–1492.

Merritt, D. J., Hawken, A., and Whitington, P. M. (1993). The Role Of the Cut Gene In the Specification Of Central Projections By Sensory Axons In Drosophila. Neuron *10*, 741-752.

Merritt, D. J., and Murphey, R. K. (1992). Projections Of Leg Proprioceptors Within the Cns Of the Fly Phormia In Relation to the Generalized Insect Ganglion. Journal Of Comparative Neurology *322*, 16-34.

Merritt, D. J., and Whitington, P. M. (1995). Central projections of sensory neurons in the Drosophila embryo correlate with sensory modality, soma position, and proneural gene-function. Journal of Neuroscience *15*, 1755-1767.

Mitchison, T., and Kirschner, M. (1988). Cytoskeletal Dynamics and Nerve Growth. Neuron *1*, 761-772.

Muller, H. A. J., and Wieschaus, E. (1996). armadillo, bazooka, and stardust are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in Drosophila. Journal of Cell Biology *134*, 149-163.

Murphey, R. K. (1985). Competition and Chemoaffinity In Insect Sensory Systems. Trends In Neurosciences *8*, 120-125.

Murphey, R. K., Bacon, J. P., and Johnson, S. E. (1985). Ectopic Neurons and the Organization Of Insect Sensory Systems. Journal Of Comparative Physiology A-Sensory Neural and Behavioral Physiology *156*, 381-389.

Murphey, R. K., Bacon, J. P., Sakaguchi, D. S., and Johnson, S. E. (1983a). Transplantation Of Cricket Sensory Neurons to Ectopic Locations - Arborizations and Synaptic Connections. Journal Of Neuroscience *3*, 659-672.

Murphey, R. K., Jacklett, A., and Schuster, L. (1980). A Topographic Map of Sensory Cell Terminal Arborizations in the Cricket CNS: Correlation with Birthday and Position in a Sensory Array. Journal of Comparative Neurology *191*, 53-64.

Murphey, R. K., Johnson, S. E., and Sakaguchi, D. S. (1983b). Anatomy and Physiology Of Supernumerary Cercal Afferents In Crickets - Implications For Pattern-Formation. Journal Of Neuroscience *3*, 312-325.

Murphey, R. K., and Lemere, C. (1984). Competition Controls the Growth Of an Identified Axonal Arborization. Science *224*, 1352-1355.

Murphey, R. K., Possidente, D., Pollack, G., and Merritt, D. J. (1989a). Modality-Specific Axonal Projections In the Cns Of the Flies Phormia and Drosophila. Journal Of Comparative Neurology *290*, 185-200.

Murphey, R. K., Possidente, D. R., Vandervorst, P., and Ghysen, A. (1989b). Compartments and the Topography Of Leg Afferent-Projections In Drosophila. Journal Of Neuroscience *9*, 3209-3217.

Muskavitch, M. A. T. (1994). Delta-Notch Signaling and Drosophila Cell Fate Choice. Developmental Biology *166*, 415-430.

Newland, P. L. (1991). Morphology and Somatotopic Organization Of the Central Projections Of Afferents From Tactile Hairs On the Hind Leg Of the Locust. Journal Of Comparative Neurology *312*, 493-508.

Newland, P. L., and Burrows, M. (1994). Processing Of Mechanosensory Information From Gustatory Receptors On a Hind Leg Of the Locust. Journal Of Comparative Physiology a-Sensory Neural and Behavioral Physiology *174*, 399-410.

Newsome, T. P., Schmidt, S., Dietzl, G., Keleman, K., Asling, B., Debant, A., and Dickson, B. J. (2000). Trio combines with Dock to regulate Pak activity during photoreceptor axon pathfinding in Drosophila. Cell *101*, 283-294.

Nolo, R., Abbott, L. A., and Bellen, H. J. (2000). Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in Drosophila. Cell *102*, 349-362.

Nose, A., Takeichi, M., and Goodman, C. S. (1994). Ectopic expression of connectin reveals a repulsive function during growth cone guidance and synapse formation. Neuron *13*, 525-539.

Nottebohm, E., Dambly-Chaudiere, C., and Ghysen, A. (1992). Connectivity Of Chemosensory Neurons Is Controlled By the Gene Poxn In Drosophila. Nature *359*, 829-832.

Nüsslein-Volhard, C., Wieschaus, E., and Kluding, H. (1984). Mutations Affecting the Pattern of the Larval Cuticle in Drosophila-Melanogaster .1. Zygotic Loci on the 2nd Chromosome. Wilhelm Rouxs Archives of Developmental Biology *193*, 267-282.

O'Dell, K. M. C., Armstrong, J. D., Yang, M. Y., and Kaiser, K. (1995). Functional Dissection Of the Drosophila Mushroom Bodies By Selective Feminization Of Genetically Defined Subcompartments. Neuron *15*, 55-61.

O'Keefe, L., Dougan, S. T., Gabay, L., Raz, E., Shilo, B. Z., and DiNardo, S. (1997). Spitz and Wingless, emanating from distinct borders, cooperate to establish cell fate across the Engrailed domain in the Drosophila epidermis. Development *124*, 4837-4845.

O'Connor, T. P., and Bentley, D. (1993). Accumulation of Actin in Subsets of Pioneer Growth Cone Filopodia in Response to Neural and Epithelial Guidance Cues in-Situ. Journal of Cell Biology *123*, 935-948.

Okabe, M., and Okano, H. (1997). Two-step induction of chordotonal organ precursors in Drosophila embryogenesis. Development *124*, 1045-1053.

Oland, L. A., Pott, W. M., Higgins, M. R., and Tolbert, L. P. (1998). Targeted ingrowth and glial relationships of olfactory receptor axons in the primary olfactory pathway of an insect. Journal of Comparative Neurology *398*, 119-138.

Oldfield, B. P. (1982). Tonotopic Organization Of Auditory Receptors In Tettigoniidae (Orthoptera, Ensifera). Journal Of Comparative Physiology *147*, 461-469.

Oldfield, B. P. (1983). Central Projections Of Primary Auditory Fibers In Tettigoniidae (Orthoptera, Ensifera). Journal Of Comparative Physiology *151*, 389-395.

Orlando, V., Jane, E. P., Chinwalla, V., Harte, P. J., and Paro, R. (1998). Binding of Trithorax and Polycomb proteins to the bithorax complex: dynamic changes during early Drosophila embryogenesis. Embo Journal *17*, 5141-5150.

Patel, N. H., Snow, P. M., and Goodman, C. S. (1987). Characterization and Cloning Of Fasciclin-Iii - a Glycoprotein Expressed On a Subset Of Neurons and Axon Pathways In Drosophila. Cell *48*, 975-988.

Patel, N. H., Schafer, B., Goodman, C. S., and Holmgren, R. (1989). The Role Of Segment Polarity Genes During Drosophila Neurogenesis. Genes & Development *3*, 890-904.

Patel, N. H. (1994). Imaging neuronal subsets and other cell-types in whole-mount Drosophila embryos and larvae using antibody probes. Methods In Cell Biology *44*, 445-487.

Perrimon, N. (1998). Creating mosaics in Drosophila. International Journal of Developmental Biology *42*, 243-247.

Pflüger, H. J. (1980). The function of hair sensilla on the locust's leg: the role of tibial hairs. Journal of Experimental Biology *87*, 163-175.

Pflüger, H. J., Braunig, P., and Hustert, R. (1981). Distribution and Specific Central Projections Of Mechanoreceptors In the Thorax and Proximal Leg Joints Of Locusts .2. the External Mechanoreceptors - Hair Plates and Tactile Hairs. Cell and Tissue Research *216*, 79-96.

Pflüger, H. J., Braunig, P., and Hustert, R. (1988). The Organization Of Mechanosensory Neuropiles In Locust Thoracic Ganglia. Philosophical Transactions Of the Royal Society Of London Series B- Biological Sciences *321*, 1.

Phelps, C. B., and Brand, A. H. (1998). Ectopic gene expression in Drosophila using GAL4 system. Methods-a Companion to Methods in Enzymology *14*, 367-379.

Prokopenko, S. N., He, Y. C., Lu, Y., and Bellen, H. J. (2000). Mutations affecting the development of the peripheral nervous system in Drosophila: A molecular screen for novel proteins. Genetics *156*, 1691-1715.

Ramain, P., Heitzler, P., Haenlin, M., and Simpson, P. (1993). Pannier, a Negative Regulator Of Achaete and Scute In Drosophila, Encodes a Zinc-Finger Protein With Homology to the Vertebrate Transcription Factor Gata-1. Development *119*, 1277-1291.

Raper, J. A., Bastiani, M., and Goodman, C. S. (1983a). Pathfinding By Neuronal Growth Cones In Grasshopper Embryos .2. Selective Fasciculation Onto Specific Axonal Pathways. Journal Of Neuroscience *3*, 31-41.

Raper, J. A., Bastiani, M. J., and Goodman, C. S. (1983b). Guidance Of Neuronal Growth Cones - Selective Fasciculation In the Grasshopper Embryo. Cold Spring Harbor Symposia On Quantitative Biology *48*, 587-598.

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

Retaux, S., McNeill, L., and Harris, W. A. (1996). Engrailed, retinotectal targeting, and axonal patterning in the midbrain during Xenopus development: An antisense study. Neuron *16*, 63-75.

Rheinlander, J. (1975). Transmission of acoustic information at three neuronal levels in the auditory system of Decticus verrucivorus (Tettigoniidae: Orthoptera). Journal of Comparative Physiology *97*, 1-53.

Ringrose, L., and Paro, R. (2001). Remembering silence. Bioessays *23*, 566-570.

Rodriguez, I., Hernandez, R., Modolell, J., and Ruizgomez, M. (1990). Competence to Develop Sensory Organs Is Temporally and Spatially Regulated in Drosophila Epidermal Primordia. Embo Journal *9*, 3583-3592.

Romani, S., Campuzano, S., Macagno, E. R., and Modolell, J. (1989). Expression Of Achaete and Scute Genes In Drosophila Imaginal Disks and Their Function In Sensory Organ Development. Genes & Development *3*, 997-1007.

Romani, S., Campuzano, S., and Modolell, J. (1987). The Achaete-Scute Complex Is Expressed In Neurogenic Regions Of Drosophila Embryos. Embo Journal *6*, 2085-2092.

Römer, H. (1983). Tonotopic Organization Of the Auditory Neuropil In the Bushcricket Tettigonia-Viridissima. Nature *306*, 60-62.

Rushlow, C. A., Hogan, A., Pinchin, S. M., Howe, K. M., Lardelli, M., and Ish-Horowicz, D. (1989). The Drosophila Hairy Protein Acts in Both Segmentation and Bristle Patterning and Shows Homology to N-Myc. Embo Journal *8*, 3095-3103.

Salzberg, A., Develyn, D., Schulze, K. L., Lee, J. K., Strumpf, D., Tsai, L., and Bellen, H. J. (1994). Mutations affecting the pattern of the PNS in Drosophila reveal novel aspects of neuronal development. Neuron *13*, 269-287.

Salzberg, A., Prokopenko, S. N., He, Y. C., Tsai, P., Pal, M., Maroy, P., Glover, D. M., Deak, P., and Bellen, H. J. (1997). P-element insertion alleles of essential genes on the third chromosome of Drosophila melanogaster mutations affecting embryonic PNS development. Genetics *147*, 1723-1741.

Saurin, A. J., Shao, Z. H., Erdjument-Bromage, H., Tempst, P., and Kingston, R. E. (2001). A Drosophila Polycomb group complex includes Zeste and dTAFII proteins. Nature *412*, 655-660.

Schrader, S., and Merritt, D. J. (2000). Central projections of Drosophila sensory neurons in the transition from embryo to larva. Journal of Comparative Neurology *425*, 34-44.

Sepp, K. J., and Auld, V. J. (1999). Conversion of lacZ enhancer trap lines to GAL4 lines using targeted transposition in Drosophila melanogaster. Genetics *151*, 1093-1101.

Shepherd, D., and Murphey, R. K. (1986). Competition Regulates the Efficacy Of an Identified Synapse In Crickets. Journal Of Neuroscience *6*, 3152-3160.

Shepherd, D., and Smith, S. A. (1996). Central projections of persistent larval sensory neurons prefigure adult sensory pathways in the CNS of Drosophila. Development *122*, 2375-2384.

Siegfried, E., Wilder, E. L., and Perrimon, N. (1994). Components of Wingless Signaling in Drosophila. Nature *367*, 76-80.

Siegler, M. V. S., and Jia, X. X. (1999). Engrailed negatively regulates the expression of cell adhesion molecules connectin and neuroglian in embryonic Drosophila nervous system. Neuron *22*, 265-276.

Sink, H., and Whitington, P. M. (1991). Early Ablation Of Target Muscles Modulates the Arborisation Pattern Of an Identified Embryonic Drosophila Motor Axon. Development *113*, 701-707.

Skeath, J. B., and Carroll, S. B. (1991). Regulation Of Achaete-Scute Gene-Expression and Sensory Organ Pattern-Formation In the Drosophila Wing. Genes & Development *5*, 984-995.

Skeath, J. B., and Carroll, S. B. (1992). Regulation Of Proneural Gene-Expression and Cell Fate During Neuroblast Segregation In the Drosophila Embryo. Development *114*, 939-946.

Skeath, J. B., Panganiban, G., Selegue, J., and Carroll, S. B. (1992). Gene-Regulation In 2 Dimensions - the Proneural Achaete and Scute Genes Are Controlled By Combinations Of Axis-Patterning Genes Through a Common Intergenic Control Region. Genes & Development *6*, 2606-2619.

Smith, S. A., and Shepherd, D. (1996). Central afferent projections of proprioceptive sensory neurons in Drosophila revealed with the enhancer-trap technique. Journal of Comparative Neurology *364*, 311-323.

Snow, P. M., Bieber, A. J., and Goodman, C. S. (1989). Fasciclin-III - a Novel Homophilic Adhesion Molecule In Drosophila. Cell *59*, 313-323.

Sorger, P. K. (1991). Heat-Shock Factor and the Heat-Shock Response. Cell *65*, 363-366.

Speicher, S., Garcia-Alonso, L., Carmena, A., Martin-Bermudo, M. D., de la Escalera, S., and Jimenez, F. (1998). Neurotactin functions in concert with other identified CAMs in growth cone guidance in Drosophila. Neuron *20*, 221-233.

Sperry, R. W. (1963). Chemoaffinity in the orderly growth of nerve fiber patterns and connections. Proceedings of the National Academy of Sciences USA *50* 703-710.

St-Johnston, D., and Nüsslein-Volhard, C. (1992). The Origin of Pattern and Polarity in the Drosophila Embryo. Cell *68*, 201-219.

Strausfeld, N. J. (1976). Atlas of an Insect Brain (Berlin, Springer).

Struhl, G., and Basler, K. (1993). Organizing Activity of Wingless Protein in Drosophila. Cell *72*, 527-540.

Strutt, H., Cavalli, G., and Paro, R. (1997). Co-localization of Polycomb protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic gene expression. Embo Journal *16*, 3621-3632.

Strutt, H., and Paro, R. (1997). The polycomb group protein complex of Drosophila melanogaster has different compositions at different target genes. Molecular and Cellular Biology *17*, 6773-6783.

Sweeney, S. T., Broadie, K., Keane, J., Niemann, H., and O'Kane, C. J. (1995). Targeted Expression Of Tetanus Toxin Light-Chain In Drosophila Specifically Eliminates Synaptic Transmission and Causes Behavioral Defects. Neuron *14*, 341-351.

Tang, A. H., Neufeld, T. P., Rubin, G. M., and Muller, H. A. J. (2001). Transcriptional regulation of cytoskeletal functions and segmentation by a novel maternal pair-rule gene, lilliputian. Development *128*, 801-813.

Tear, G., Harris, R., Sutaria, S., Kilomanski, K., Goodman, C. S., and Seeger, M. A. (1996). commissureless controls growth cone guidance across the cns midline in Drosophila and encodes a novel membrane-protein. Neuron *16*, 501-514.

Tessier-Lavigne, M., and Goodman, C. S. (1996). The molecular-biology of axon guidance. Science *274*, 1123-1133.

Tix, S., Bate, M., and Technau, G. M. (1989a). Pre-existing neuronal pathways in the leg imaginal disks of Drosophila. Development *107*, 855-862.

Tix, S., Minden, J. S., and Technau, G. M. (1989b). Pre-existing neuronal pathways in the developing optic lobes of Drosophila. Development *105*, 739.

Usui-Ishihara, A., Simpson, P., and Usui, K. (2000). Larval multidendrite neurons survive metamorphosis and participate in the formation of imaginal sensory axonal pathways in the notum of Drosophila. Developmental Biology *225*, 357-369.

Vaessin, H., Grell, E., Wolff, E., Bier, E., Jan, L. Y., and Jan, Y. N. (1991). Prospero Is Expressed In Neuronal Precursors and Encodes a Nuclear- Protein That Is Involved In the Control Of Axonal Outgrowth In Drosophila. Cell *67*, 941-953.

Van Doren, M., Bailey, A. M., Esnayra, J., Ede, K., and Posakony, J. W. (1994). Negative Regulation of Proneural Gene Activity - Hairy Is a Direct Transcriptional Repressor of Achaete. Genes & Development *8*, 2729-2742.

Van Doren, M., Powell, P. A., Pasternak, D., Singson, A., and Posakony, J. W. (1992). Spatial Regulation of Proneural Gene Activity - Autoactivation and Cross-Activation of Achaete Is Antagonized by Extramacrochaetae. Genes & Development *6*, 2592-2605.

Vervoort, M., Merritt, D. J., Ghysen, A., and Dambly-Chaudiere, C. (1997). Genetic basis of the formation and identity of type I and type II neurons in Drosophila embryos. Development *124*, 2819-2828.

Vervoort, M., Zink, D., Pujol, N., Victoir, K., Dumont, N., Ghysen, A., and Dambly-Chaudiere, C. (1995). Genetic-Determinants Of Sense Organ Identity In Drosophila - Regulatory Interactions Between Cut and Poxn. Development *121*, 3111-3120.

Vincent, J. P., and O'Farrell, P. H. (1992). The State of Engrailed Expression Is Not Clonally Transmitted During Early Drosophila Development. Cell *68*, 923-931.

Walthall, W. W., and Murphey, R. K. (1984). Rules For Neural Development Revealed By Chimaeric Sensory Systems In Crickets. Nature *311*, 57-59.

Weiss, J. B., Von Ohlen, T., Mellerick, D. M., Dressler, G., Doe, C. Q., and Scott, M. P. (1998). Dorsoventral patterning in the Drosophila central nervous system: the intermediate neuroblasts defective homeobox gene specifies intermediate column identity. Genes & Development *12*, 3591-3602.

White, B. H., Osterwalder, T. P., Yoon, K. S., Joiner, W. J., Whim, M. D., Kaczmarek, L. K., and Keshishian, H. (2001). Targeted attenuation of electrical activity in Drosophila using a genetically modified K+ channel. Neuron *31*, 699-711.

White, K. P., Rifkin, S. A., Hurban, P., and Hogness, D. S. (1999). Microarray analysis of Drosophila development during metamorphosis. Science *286*, 2179-2184.

Wieschaus, E., and Nüsslein-Volhard, C. (1998). Looking at Embryos. In Drosophila: A Practical Approach, D. B. Roberts, ed. (Oxford: IRL), pp. 179-213.

Wieschaus, E., Nüsslein-Volhard, C., and Jurgens, G. (1984). Mutations Affecting the Pattern of the Larval Cuticle in Drosophila-Melanogaster .3. Zygotic Loci on the X-Chromosome and 4th Chromosome. Wilhelm Rouxs Archives of Developmental Biology *193*, 296-307.

Williams, D. W., and Shepherd, D. (1999). Persistent larval sensory neurons in adult Drosophila melanogaster. Journal of Neurobiology *39*, 275-286.

Williams, D. W., Tyrer, M., and Shepherd, D. (2000). Tau and tau reporters disrupt central projections of sensory neurons in Drosophila. Journal of Comparative Neurology *428*, 630-640.

Williams, D. W., and Shepherd, D. (2002). Larval sensory neurones guide adult axon growth. Development *129* (in press).

Xu, T., and Harrison, S. D. (1994). Mosaic Analysis Using Flp Recombinase. Methods In Cell Biology *44*, 655-681.

Xu, T., and Rubin, G. M. (1993). Analysis of Genetic Mosaics in Developing and Adult Drosophila Tissues. Development *117*, 1223-1237.

Yang, M. Y., Armstrong, J. D., Vilinsky, I., Strausfeld, N. J., and Kaiser, K. (1995). Subdivision of the Drosophila Mushroom Bodies By Enhancer-Trap Expression Patterns. Neuron *15*, 45-54.

Yu, H. H., Araj, H. H., Ralls, S. A., and Kolodkin, A. L. (1998). The transmembrane semaphorin Sema I is required in Drosophila for embryonic motor and CNS axon guidance. Neuron *20*, 207-220.

Zinn, K., McAllister, L., and Goodman, C. S. (1988). Sequence-Analysis and Neuronal Expression Of Fasciclin-I In Grasshopper and Drosophila. Cell *53*, 577-587.

zur Lage, P., Jan, Y. N., and Jarman, A. P. (1997). Requirement for EGF receptor signalling in neural recruitment during formation of Drosophila chordotonal sense organ clusters. Current Biology *7*, 166-175.