From Embryo to Adult: Anatomy and Development of a Leg Sensory Organ in *Phormia regina*, Meigen (Insecta: Diptera). II. Development and Persistence of Sensory Neurons

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ABSTRACT

The imaginal leg disc of *Phormia regina* contains eight neurons that arise during embryogenesis. Five of the neurons are associated with Keilin's organ, and of these five, two persist to the adult fly. Two new neurons arise at about the time of pupariation and flank each of these persisting neurons, forming two triplets of cells. Both triplets can be followed throughout metamorphosis; in the late pupa they are situated anteriorly and posteriorly at the tip of the fifth tarsomere. Two triplets of cuticular specializations are found at corresponding positions in the adult fly, each consisting of two campaniform sensilla and a trichoid hair. The central member of each set of sensilla, a campaniform sensillum, is associated with the persisting cell.

Key words: metamorphosis, imaginal disc, immunocytochemistry, blowfly, mechanoreceptor

In the preceding paper (Lakes-Harlan et al., '91) we showed that five of the eight neurons found in the imaginal leg discs of blowflies are associated with a larval sense organ, Keilin's organ. Three of these neurons terminate at the bases of the three hairs of the organ and appear to be mechanoreceptors. The remaining two neurons terminate at the cuticle. Neither of these is associated with any obvious cuticular specialization, and one retracts from the cuticle before the larval stage is complete. Unlike the dendrites that are associated with hairs, neither of these has a well-developed tubular body. Thus, in contrast to the remaining three cells, the morphological features of these two cells do not strongly suggest that they serve as larval sensory neurons. These dendrites are morphologically similar, in the absence of a cuticular termination and of a well-developed tubular body, to dendrites of developing sensory neurons (Ameismeier, '85).

It has previously been shown that some neurons of the imaginal disc remain in the developing leg (Jan et al., '85; Lakes et al., '89; Tix et al., '89b). Persistence of larval sensory neurons to the late pupa has also been reported in *Manduca* (Bate, '73; Levine et al., '85; Levine, '89). In the present paper we trace the neurons of the imaginal leg disc of the blowfly through development from the embryo through the adult, and show that two of the cells that are

associated with Keilin's organ persist throughout development.

MATERIALS AND METHODS

The methods were described in the preceding paper (Lakes-Harlan et al., '91). In order to stain neurons in late pupal legs (after formation of the antibody-impermeant adult cuticle) longitudinal incisions were made to allow antibodies access to the legs' interiors, and the durations of all incubation times were tripled. In order to be certain that we did not miss important developmental events, animals were collected at 6 hour intervals (6 hours = 2.7% of larval development) during the transition from larva to pupa. During the initial phases of pupal development, when changes occur rapidly, the intervals were less than 0.5 hours (Lakes and Pollack, '90).

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Fig. 1. Origin of the neurons in the imaginal leg disc. Anti-HRPlabeled neurons in wholemounts of embryos and larvae of *Phormia regina*. **a**: Sensory neurons situated ventrally in the prothoracic segment of an embryo 10.5 hours after egg laying (corresponding to stage 16 in *Drosophila*; Campos-Ortega and Hartenstein, 1985). d, tips of dendrites; mvn, medioventral nerve; vc, vc', two separate clusters of ventral sensory neurons; tn, thoracic nerve; the arrowhead points to a dendrite of the md cell of the vc cluster. Nomarski optics. Scale bar = 20 μ m. **b**: Invaginating prothoracic leg discs in an early third instar larva. The neurons of the vc, as well as that of one of the campaniform sensilla

RESULTS

Origin of sensory neurons in the imaginal leg disc

In embryos two clusters of cells (vc, vc'; Ghysen et al., '86 for *Drosophila*), which total 17–18 sensory neurons, are found ventrally in each thoracic segment (Fig. 1a). The vc' cluster consists of five neurons with multiply branching dendrites (md-neurons; Bodmer and Jan, '87), three bipolar neurons associated with a basiconical sensillum, and two bipolar neurons that innervate two campaniform sensilla. of the vc' can be seen; the remaining vc' neurons are out of the field. cs, campaniform sensilla; Ko, Keilin's organ; tp, tracheal process. Scale bar = 20 μ m. c: Drawing of an invaginated mesothoracic leg disc shortly after the stage shown in b. The neurons of the vc' cluster, which remain at the cuticle, are stippled. bc, neurons of the basiconical sensillum; hs, hypodermal stalk connecting the disc to the cuticle; md, multiple dendrite cell; bp, bipolar cell; mo, axons of two motor neurons that run in the mvn; sc, scolopidial sensillum. Scale bar = 40 μ m. d: Lateral view of a leg disc with the connections to the cuticle and the central nervous system (CNS). pc, peripodial cavity. Scale bar = 200 μ m.

The vc cluster is a dense group of cells between the two campaniform sensilla, containing six bipolar neurons and at least one md neuron (Fig. 1a). Five of the bipolar neurons are associated with the Keilin's organ (Keilin, '15; Lakes-Harlan et al., '91) and the sixth is associated with a scolopidium. In first instar larvae the cell bodies of the neurons of the vc cluster are embedded in presumptive imaginal disc tissue, which can be distinguished from surrounding tissue because it is composed of small cells with small nuclei (Madhavan and Schneiderman, '77). Soon after the molt to the third larval instar the disc, including

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Fig. 2. Anti-HRP-labeled neurons of the leg disc of a third instar larva of *Phormia regina*. Anterior is to the top; Nomarski optics. Scale bar = $20 \ \mu$ m. **a**: Scolopidial sensory organ at the dorsal surface of the disc. cb, cell body of the sensory neuron; cc, cap cell; d, dendrite. **b**: Neuron with a multiply branching dendrite (two branches are in focus) on the dorsal surface of the disc. **c**: Cell bodies of the bipolar neurons of the anterior group.

the dense group of neurons (vc) described above, invaginates into the body cavity.

Neurons within the imaginal leg disc

Anatomical relationship between imaginal leg disc and the larval nervous system

Each leg disc, after having invaginated, remains connected to the larval cuticle by a hypodermal stalk, which terminates peripherally at Keilin's organ, and a tracheal process. The stalk contains the dendrites of the cells that are associated with Keilin's organ; these dendrites elongated as their somata moved into the interior of the larva with the rest of the disc. The axons of the neurons within the disc project to the CNS through a nerve that exits the disc proximally (Fig. 1c,d). The somata of the neurons of the vc' cluster remain in the usual location for insect sensory neurons, just beneath the cuticle.

The nerve that connects the disc to the CNS carries, in addition to the axons of the neurons found within the disc, the axons of at least two motor neurons. These motor neurons can be identified as such by their centrally located somata and dendritic trees (Lakes-Harlan et al., '91). The peripheral nerves are formed during embryogenesis (see Hartenstein, '88 for *Drosophila*) and the imaginal leg disc is integrated within this existing system when it invaginates. Both prior to and after disc invagination, the axons of the vc' cells travel together to the CNS in the posterior nerve, which is a branch of the thoracic nerve (Fig. 1a). The vc axons run in a separate medioventral nerve [Fig. 1a; Hartenstein ('88) referred to this as a branch of the posterior nerve]; it is this nerve that, after invagination, forms the connection between the disc and the CNS. In late third instar larvae eight neurons are found in each leg disc. At least seven of these are identical with the neurons in the vc group described in the embryo; the origin of the eighth cell is not yet certain. The neurons can be subdivided into two groups; three neurons are near the dorsal surface of the disc and the remaining five have their somata in the disc epithelium. One of the dorsal neurons is associated with a scolopidium (Fig. 2a; see also van Ruiten and Sprey, '74 for *Calliphora*). The other two dorsal neurons have multiply branching dendrites that cover much of the anterior, dorsal surface of the disc (Fig. 2b). In recently invaginated discs, some of these dendrites proceed into the tracheal process.

The five neurons of the epithelial layer can be further subdivided into an anterior group of four cells and a solitary posterior cell. All five neurons have dendrites that extend into the peripodial cavity (Fig. 2c). Within the anterior group consistent morphological differences are found. The somata of two of the cells are closely apposed, and their axons run in an anterior bow to the larval nerve. A third soma is relatively close but its axon follows a different, more direct course to the nerve. The axon of the fourth cell, the soma of which is the most posterior within the anterior group, follows the path of the first two cells. Its dendrite exhibits a posterior-medial loop before it joins those of the other anterior cells en route to the hypodermal stalk. The dendrites of the four anterior cells can be seen, in all preparations stained with anti-horseradish peroxidase (HRP), to enter the peripodial cavity of the leg disc and proceed down the hypodermal stalk (Lakes-Harlan et al.,

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'91). In preparations from third instar larvae, the dendrite of the posterior cell can only rarely be seen to enter the stalk; more typically it ends within the peripodial cavity of the disc proper.

Rearrangement of neurons during metamorphosis

The imaginal leg discs give rise to the adult legs during the pupal stage. At about the time of pupariation six of the eight neurons of the disc appear to degenerate (Lakes and Pollack, '90). The remaining two neurons persist. One of these is the posterior cell of the epithelial cell layer, and the other is one of the group of four anterior cells in the epithelial layer, specifically, the cell with the more posteriorly situated soma and posterior-medial looping dendrite (Fig. 3a). Both of these persistent cells become flanked by two newly appearing neighbors. The sequence of events is similar near both the anterior and the posterior cell. First, a new sensory cell arises posterior to the persisting one (this will correspond to a more dorsal position in the adult leg) at about 91% of the larval development, i.e., about 18 hours before pupariation (Fig. 3a-d). A characteristic sequence of immunocytochemical events foretells the appearance of this cell. First a broad patch of the surface of the epidermis is labeled by the anti-HRP antibody. Then a group of two to three adjacent cells, which span the thickness of the epithelial layer, appears below the patch (Fig. 3c,d). These cells can be stained with anti-HRP for a period of approximately 12 hours, but after this only a single sensory neuron is labeled. Its axon grows towards the larval nerve, and the patch of label at the surface of the epidermis narrows, and marks the apical end of the dendrite. At the time it first appears, this new neuron is at a distance of 48 µm (SD = 7.0, n = 22) from the persisting cell. By the time of pupariation this distance has decreased to $14 \mu m$ (SD = 4.3, n = 10), and by 40 hours it has decreased to 6 μ m (SD = 0.9, n = 14). A third bipolar neuron appears anterior to the persisting cell (corresponding to a more ventral position in the adult leg) at about the onset of pupariation (Fig. 3e).

Thus, shortly after pupariation there are two triplets of cells, one anterior and one posterior (Fig. 3f,g). The central member of each triplet is a persistent, larval neuron, and the outer members are new additions. In early pupae the central cells differ morphologically from their younger neighbors. They have greatly elongated dendrites, which run along the outer surface of the metamorphosing disc. These are remnants of the elongated dendrites that, in the larva, extended down the hypodermal stalk to Keilin's organ (Lakes-Harlan et al., '91). The dendrite of the anterior persisting cell can be seen, in early pupae, to run parallel to the similarly long dendrites of the other members of the anterior group of epithelial cell layer neurons of the larval disc, which are at this time in the process of degeneration (Lakes and Pollack, '90). In addition, the somata of the central cells of the triplets are closer to the basal boundary of the epithelial cell layer than those of their partners (Fig. 3b,c) and stain more darkly.

The two triplets of cells, and particularly their parallel dendrites, are conspicuous features of the developing limb, and can be recognized unambiguously throughout pupal development. They are situated in the distalmost tarsomere. By 55 hours of pupal development the gross morphology of the tarsus is well established, and the dendrites of the triplets can be seen to end laterally at the distal end of the fifth tarsomere (Fig. 3g). Inspection of this region of the tarsus of adults with scanning electron microscopy reveals the three sensilla that are associated with the triplets of cells. The most lateral sensillum is a short (12 μ m) trichoid hair (Fig. 4a,b). More medially are two campaniform sensilla. The dome (5 μ m diameter) of the one nearest to the hair has a distinct rim, while the dome (8 μ m diameter) of the one nearest to the midline of the leg is more or less continuous with the surrounding cuticle (Fig. 4b). The innervation of these structures by the neurons of the triplets can be seen in 3 day old pupae, shortly after the cuticular structures first appear (Fig. 3h).

DISCUSSION

In this paper we have shown that two sensory neurons persist from the embryo to the adult in the blowfly, Phormia regina (Fig. 5). We showed previously that the imaginal leg discs of Drosophila are similar in many respects to those of Phormia, including the occurrence of neurons that project to Keilin's organ, and the occurrence of anterior and posterior triplets in the distalmost tarsomere of developing pupal legs (Lakes and Pollack, '90). It seems likely that, as in Phormia, the central member of each of these triplets is a persistent neuron that was born during embryogenesis. Three cuticular structures, similar to those we describe here for Phormia, are also seen with scanning electron microscopy on the anterior and posterior faces of the distal tarsus of Drosophila (Merritt and Murphey, in preparation). Thus, the similarities that have previously been noted between the peripheral organization and central projections of leg sensory neurons in adults of these two species (Murphey et al., '89) and the postembryonic development of these neurons (Lakes and Pollack, '90) are likely to extend to the persistence of a subset of larval neurons.

Recently, Tix et al. ('89b) demonstrated, using a different technique, that neurons found in leg discs of Drosophila arise early in embryonic development. They injected HRP into embryos before cellularization, and later identified those cells that still had relatively high concentrations of HRP, and therefore had undergone few cycles of growth and division since the injection. Some of the cells they describe are similar to those we find in the discs of *Phormia*, but we find some differences as well. The group of cells they identify as S3 corresponds to the three anteriormost cells we observe in the anterior compartment of the epithelial layer of the disc (together with their nonneural support cells). Their S2 group appears to correspond, in position within the disc and in dendrite trajectory, to the posteriormost member of the anterior epithelial layer group of Phormia, i.e., the cell that persists as the central member of the anterior triplet. Tix et al. ('89b) report two neurons in this group in Drosophila, but using anti-HRP staining we find only one neuron here, both in Phormia and Drosophila (Lakes and Pollack, '90). S1 of Drosophila corresponds to the posterior epithelial layer cell of Phormia, and S4 corresponds to the scolopidial cell. Tix et al. also found that several cells in the basal region of the disc arise early in development, and identified these as adepithelial cells (muscle precursors). We find two multiple-dendrite neurons in this region in Phormia, and one in Drosophila.

An interesting question arises concerning the relationship between the persisting neurons and their associated





Fig. 3. Anti-HRP-labeled neurons during metamorphosis of the leg disc. **a:** Dorsal overview of the imaginal leg disc (92% of larval development). The arrow points to the newly arisen cells. as, seperated cell of the anterior group (a); d, dendrites of the neurons of the anterior group; p, posterior cell; proximal is to the left. Scale bar = 50 μ m. **b:** Lateral view of the posterior cell and the newly arisen neuron (96%). pc, peripodial cavity; Scale bar = 50 μ m. **c:** Ventrolateral view of the anterior group with the newly arisen cell (92%). Scale bar = 50 μ m. **d:** Higher magnification of the developing neuron, showing the temporary staining of two cell bodies with the anti-HRP antibody (94%). Scale bar = 10 μ m. **e:** Tip of the fifth tarsomere (0.5 hours after the onset of pupariation). A third neurons arises (arrow) near the distal end of the persisting neuron. Scale bar = 50 μ m. **f:** The tip of a fifth tarsomere, showing the anterior triplet of neurons (5 hours after pupariation). The posterior triplet is out of focus. Scale bar = 10 μ m. **g:** The fifth tarsomer of a late pupa (55 hours), viewed dorsolaterally. The arrow points to one of the triplets of cells. Scale bar = 50 μ m. **h:** Termination of the posterior triplet of neurons at the bases of cuticular structures (72 hours after pupation). cs, campaniform sensilla; tr, trichoid hair. Scale bar = 10 μ m.



Fig. 4. Scanning electron micrographs of the fifth tarsomere of the adult foreleg. **a:** Overview of the fifth tarsomere. p, pulvillus; c, terminal claw. Scale bar = $20 \ \mu m$. **b:** Larger magnification of the area marked in a showing the triplet of sensory structures. cs, campaniform sensilla; tr, trichoid hair. Scale bar = $5 \ \mu m$.

cuticular specializations. The cuticular components of sensilla are produced by auxiliary cells that are descendants of the same epithelial precursor cell as the neuron(s) (Bate, '78); if they are maintained throughout development, they form the same type of sensillum at each molt. In the larva of the blowfly, the cuticular specializations of Keilin's organ are hairs. In the adults, the persisting neurons are associated with campaniform sensilla. These sensillum types differ not only in external structure but also in modality (hairs are tactile receptors, campaniform sensilla are proprioreceptors) and in the central targets of their sensory neurons (Pflüger et al., '88; Merritt and Murphey, in preparation). If the usual relationships between neurons, auxiliary cells, and cuticular structures apply here, then how might the switch from hairs to campaniform sensilla come about? If the persistent cells are among the three that terminate at the hairs of Keilin's organ, the auxiliary cells that produce hairs during embryogenesis (and, presumably, at each of the larval molts) might become respecified at pupation to produce campaniform sensilla. If, however, the persistent cells are the two that are not associated with cuticular specializations in the larva, their associated auxiliary cells might defer the production of cuticular components until the pupal stage, at which time they would produce campaniform sensilla. One of the dendrites not associated with a hair belongs to the posterior cell of the epithelial cell layer of the disc, which we know persists, and the other to one of the anterior cells. It is tempting to speculate that it is the anterior cell with no cuticular specialization that persists, although we have no direct evidence for this. One of the anterior cells also stands out from the others in its projection to the central nervous system; it terminates more medially than the others. Again,

though we have no evidence for this as yet, it may be that the more medial terminals belong to the persistent cell.

Persistent neurons have often been described in holometabolous insects. Most of these are interneurons (Breidbach, '87a,b, '89; Cantera and Nässel '87; Nässel et al., '88; Tix et al., '89a,b) or motor neurons (Taylor and Truman, '74; Casaday and Camhi, '76; Levine and Truman, '82; Kent and Levine, '88). However, sensory neurons have been found to persist from the larva to the pupa of Manduca (Bate, '73; Levine et al., '85; Levine, '89). One of the functions that has been proposed for persistent neurons is to act as guides for later appearing neurons (Levine, '86; Tix et al., '89a,b). The axons of the persisting neurons that we have described do contribute to the major nerve pathways in the distal developing limb, which are joined by later appearing neurons (Jan et al., '85; Lakes and Pollack, '90). However, these early nerve pathways also include the axons of the other six neurons of the imaginal leg disc, all of which degenerate shortly after the onset of metamorphosis. Pioneer neurons in grasshopper limb buds also degenerate long before development is complete (Kutsch and Bentley, '87). Thus, their possible roles as pioneer neurons do not explain the persistence to adulthood of the two neurons we described. It is also clear that the later appearing neurons are able to establish their projections in the absence of obvious neural guiding structures. This is the normal situation in more proximal regions of developing fly legs (Lakes and Pollack, '90), as well as in all appendages except for the legs, since it is only in the imaginal leg discs that neurons are found in larvae (Jan et al., '85). Tix et al. ('89a) found recently that ectopic leg discs in antennapaedia mutants (i.e., antennal discs that have been homeotically transformed) lack the early neurons of normal leg discs, but that





Fig. 5. Summary of the developmental changes in neurons of the imaginal leg disc. The diagrams to the left outline the metamorphosis of the leg, from presumptive imaginal disc tissue in the embryo, to the leg proper in the pharate adult. Relative sizes of the different stages are indicated by the horizontal bars, which correspond to $250 \ \mu m$ for all stages. Triangles indicate the locations of the neurons in question. These neurons are shown on the right at higher magnification. In the embryo five neurons terminate at or near Keilin's organ. During the third larval instar the leg disc invaginates. Four of the neurons come to

lie in the anterior compartment of the disc (filled, cross-hatched), and one in the posterior compartment (stippled). By the late third instar the dendrite of the posterior cell has retracted and new cells have begun to appear (unfilled). During the early pupal stage, the two triplets of cells become established, each comprised of a persisting neuron (anterior: cross-hatched; posterior: stippled) and two new neurons. The remaining three larval neurons (filled) begin to degenerate. CNS, central nervous system; ID, imaginal disc; KO, Keilin's organ; Fe, femur; Ta, tarsus; Ti, tibia; Tr, trochanter.

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essentially normal nerve pathways nevertheless form during the pupal stage, again indicating that any pioneering function of the persisting cell is not necessary for later development.

Another interpretation stems from the consideration of the nature of holometabolic development in Diptera. The spatial and temporal separation of embryonic and postembryonic development that characterizes the holometabola varies in degree, and is extreme in flies (Anderson, '72). In Drosophila, for example, the cells that will form the imaginal discs and, eventually, adult structures, are parceled out from their neighbors very early in embryogenesis [3 hours after fertilization (Bryant and Schneiderman, '69)], but do not begin to differentiate visibly until several days later, when pupation occurs. Holometabolism is believed to be a modification of the more primitive hemimetabolous pattern, in which the basic body plan is established during embryogenesis, and is elaborated upon during later development. For the most part, the development of the sensory neurons of the legs of flies follows the dipteran norm of extreme metamorphosis; most of the larval neurons associated with the leg discs degenerate at about time of pupariation, and the vast majority of adult sensory neurons first appear in the first few days of the pupal stage (Lakes and Pollack, '90). The two persistent neurons we describe in the present paper, however, may be more "hemimetabolic" in their development; they arise during embryogenesis, but may not become functional until they acquire their cuticular apparatus during the pupal period. We cannot offer any explanation for why (assuming for the moment that the preceding interpretation is correct) these particular neurons retained their ancestral, hemimetabolic characteristics after the loss of the larval legs during evolution (Hennig, '48).

The argument just presented holds that the nonpersisting neurons in the imaginal discs are larval, rather than adult, in character. In the preceding paper (Lakes-Harlan et al., '91) we presented morphological and physiological evidence that at least some of these neurons are functional during larval life. In the present paper we showed that the imaginal leg discs, in which the neurons reside, are intimately associated with the larval nervous system and epidermis. Taken together, our findings reinforce the suggestion, first put forward by Keilin himself ('15) that Keilin's organ is the vestige of an ancestral larval leg (see also Tear et al., '88). The two persistent neurons associated with Keilin's organ come to lie in the most distal tarsomere of the adult leg. This suggests that Keilin's organ may be the remnant of specifically distal regions of the larval leg. This conclusion is strengthened by the recent observation in Drosophila that a gene necessary for normal development of distal structures of adult appendages is also required for the formation, during embryogenesis, of Keilin's organ (Sunkel and White, '87; Cohen and Jürgens, '89; Cohen et al., '89).

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