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Pathfinding by Peripheral Pioneer Neurons in Grasshoppers

David Bentley and Haig Keshishian

The precision with which connections are made between nerve cells and their targets is critical for the information processing that underlies the generation of behavior. The problem of understanding how such precision arises appears to have two aspects. How are growth cones at the tips of elongating nerve fibers able to navigate accurately, often over long distances, to the vicinity of appropriate target cells, and why are synapses established with a particular subset of the cells encountered by nerve processes? Although much is known of the behavior of growth cones in culture (*1*), long-distance pathfinding is not understood in any in vivo system.

An example of long-distance pathfinding is the formation of connections between peripheral sensory or motor elements and the central nervous system (CNS). Afferent or efferent neural growth cones must often traverse distances of hundreds or thousands of cell diameters to reach their targets. These axons do not span the distance between periphery and CNS independently but are collected into the fascicles of nerves or nerve trunks. Nerves generally have a stereotyped branching pattern so that homologous branches can be recognized in different individuals. Peripheral nerves of adult grasshoppers are arranged in this fashion (*2*). They contain the axons of both motor neurons, with cell bodies in the CNS, and sensory neurons, with cell bodies usually in the epidermis (*3*, *4*). Stereotyped nerve branching patterns and also axonal fasciculation may reflect guidance mechanisms operating on growth cones.

Analysis of grasshopper development offers a system where the entire sequence from the birth of the first neurons to the establishment of the peripheral nerve pattern can be observed at the level of single cells, and where key cells in this process are individually identifiable. In this article, we review existing information and present new data on the development of long-distance nerve pathways in the grasshopper embryo.

Grasshopper Embryogenesis

Grasshoppers offer an advantageous preparation for studying embryogenesis. Differentiating cells can be viewed in the embryo with interference contrast optics, can frequently be uniquely identi-

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fied, can be impaled with microelectrodes for dye injection and for analysis of electrophysiological properties, can be stained with neuron-binding antibodies (*5, 6*), and can be grown in embryo culture after experimental manipulations (*7, 8*). Goodman, Bate, and co-workers (*9, 10*) have provided a great deal of information on neuronal precursors and on the lineage and morphological, physiological, and biochemical differentiation of neurons in the CNS. Embryogenesis can be evaluated in the context of a wealth of data on identified neurons in the adult nervous system (*11*).

Morphogenesis of the grasshopper metathoracic leg, the focus of our attention, is first apparent at about the 25 percent stage [development is staged according to the percentage of embryogenesis completed (*12*)]. The appendage bud is a hollow evagination of the body wall surrounded by an epithelial cell monolayer, which secretes cuticle on its external surface and an acellular basement membrane on its internal surface. Inside the basement membrane, within the limb lumen, are mesodermal cells that will give rise to muscle and other internal structures (Fig. 1C). Limb elongation is accompanied by cell division, both within segments and at the tip, so that cells which are the most distal at one stage have taken on a more proximal position at later stages. As growth continues, the leg segments are delineated, intrinsic muscles are formed, and by the 55 percent stage the basic mature form of the limb is evident (Fig. 1B). Thus, the peripheral nervous system will arise within a partially hollow limb bud containing loosely arranged mesodermal cells and surrounded by epithelium.

Pioneer Neurons

Bate (*13*) drew attention to nerve formation in grasshopper appendage morphogenesis by describing the first axons that appear. Examination of electron micrographs of the metathoracic leg (and also antenna) revealed that these axons are a centripetally growing pair originating from two neurons lying distally in the lumen. These cells were designated "pioneer" neurons, a term which can usefully apply to any cell, regardless of type, whose growth cone first navigates a particular pathway. Similar neurons were described shortly thereafter in the cercal appendages of crickets (*14*); small clusters of neurons are also found connecting everted imaginal disks to the CNS in the metamorphosing insects *Manduca* and *Drosophila* (*15*).

In the grasshopper leg, the first pioneer neurons arise from a mother cell, which appears within the epithelium at the tip of the 30 percent stage limb (*16*). This cell divides symmetrically and the daughters emerge into the lumen (Fig. 1C). After a period when filopodia are extended in various directions and retracted, a process we term filopodial exploration, an axonal growth cone is projected toward the limb base along the interior surface of the epithelium (*16*) (Fig. 2, A and B). Jan and Jan (*5*) discovered that antibody against a peroxidase (horseradish peroxidase) can be used to selectively stain early neurons in *Drosophila* and grasshoppers, and similar information is provided by a monoclonal antibody developed by Chang *et al.* (*6*). Such staining confirms that the pioneer growth cones are the first to navigate through this region (Fig. 2B).

These pioneer axons do not grow in a straight trajectory from the cell body to the CNS. The growth cones extend along the anterior margin of the limb for several cell diameters and then make a sharp, posterior turn and grow on the ventral epithelium (*13, 16*) (Figs. 2A and 4A). Upon approaching the posterior margin of the limb, they make a second, sharp, medial turn and grow to the CNS (Figs. 3D and 4A). Completion of the route from the cell body to the CNS occurs by the 34 percent stage. The geometric complexity of this route provides an opportunity to search for features along the pathway which underlie guidance.

Guidepost Cells

Examining the pioneer pathway, Bate (*13*) suggested that "sheathing cells" might act as "stepping stones" for the pioneer growth cones. More recently, guidance based on contacts between specific cells has been proposed by Goodman and co-workers for axonal pathfinding within the grasshopper CNS (*9, 17*). We observe that, at three locations, a distinctive cell or cell pair is located along the pioneer pathway. Cell F1 is a large cell on the anterior margin of the lumen directly proximal to the pioneers and about 50 micrometers away at the time of pioneer axonogenesis (Figs. 3D and 4A). Cell F2 is another large cell, also on the anterior lumen margin and about 50 μm proximal to cell F1 (Figs. 2C and 3D). Cell-pair CT1 is located near the posterior margin of the limb, on the ventral surface, and about 50 μm across from cell F2 (Figs. 2B and 3, B and D). Several aspects of the relationship between these cells and the pioneers are notable.

Summary. Grasshopper neurons accurately project axons across long distances between peripheral structures and the central nervous system. Nerve-trunk pathways followed by these axons are established early in embryogenesis by pioneer neurons. Growth cones from the first pioneers navigate along a chain of cells to the CNS. The placement of these cells may constitute the initial guidance mechanism underlying long-distance pathfinding.

1) *Location.* The cells are all within the pioneer pathway. More important, F2 and CT1 are at the corners where the turns occur. The pioneers project axons as if they were growing from F1 to F2 to CT1 to the CNS.

2) *Contact.* The pioneer growth cones grow toward and contact each of these cells. This is observed by single or double intracellular dye injections (Figs. 3D, 4, A and B), or by binding fluorescently tagged antibody to the cells (Figs. 2C and 4E). Membrane apposition has been confirmed at the ultrastructural level for contact between the pioneers and cell F1 (Fig. 4D).

3) *Selective junction formation.* The pioneers form junctions, which permit the passage of molecules between cells, with each of these cells and with no other cells along the pathway. The existence of these junctions is demonstrated by intracellular injection of the fluorescent dye Lucifer yellow (molecular weight, 450). Injection of the dye into a pioneer or into any member of this set of cells it has contacted is followed by rapid diffusion of the dye molecule into all of the other cells which have been contacted (*18*) (Figs. 3D and 4, A and B). The dye does not appear in any additional cells. These nonpolarized, dye-passing junctions are first established by pioneer growth cone filopodia (*18*).

4) *Distinctiveness.* As a group, these cells are distinctive from all other cells in the pioneer pathway. Before being contacted by the pioneer growth cones, each of these cells can bind tagged antibody, and no additional cells in the limb do so (Fig. 2, B and C). This antibody binds at the cell surface (*5*). Therefore, each cell in this group displays on its surface at least one type of molecule which distinguishes it from surrounding cells and

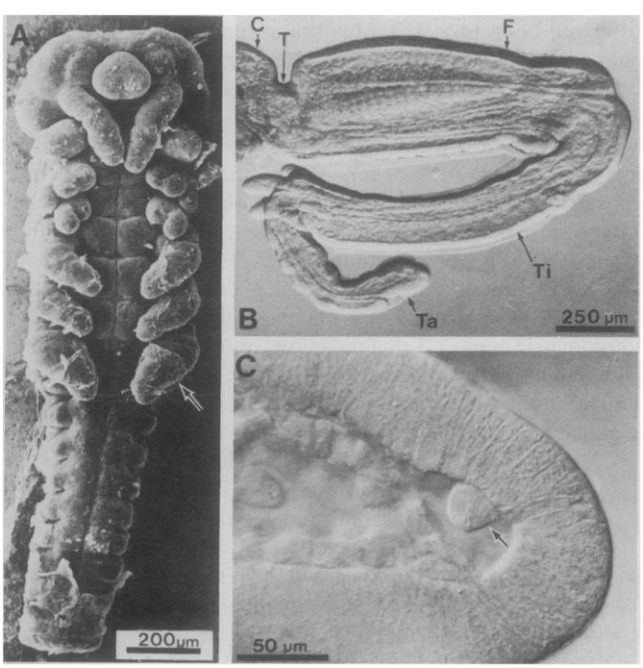
does so before arrival of the pioneer axons. The distinctiveness of these cells is confirmed by the observation that each eventually differentiates morphologically into a neuron. Cell F1 becomes a multipolar neuron after it has been contacted by the pioneers (Fig. 4, A and C), as does cell F2 (Fig. 4C). The paired CT1 cells are the second set of pioneer neurons to appear in the limb; their axons reach the CNS before the first pioneers, except in the metathorax (*13*), and the axons of the first pioneers grow along them (*19*). Selective experimental removal of the first pioneers (discussed below) demonstrates that none of these cells depend on their contact with the pioneers for subsequent axonogenesis.

5) *Spacing*. While these cells are not contiguous, they are separated by only a few cell diameters at the time when the first pioneer growth cones are navigating the pathway to the CNS. The possible significance of this distance is that it does not exceed the span of filopodial exploration from pioneer growth cones.

Taken together, these results provide substantial support for the hypothesis that the guidance mechanism for navigation of the first pioneer growth cones is the placement of a series of distinctive cells that serve as guideposts, so that the pioneers locate the CNS by growing from cell to cell along this chain. Our data show that these guidepost cells do have the characteristics which would be necessary to serve as a guidance substrate, distinctiveness and accessibility, and that the pioneers do grow along the cell chain and do establish a special and selective relationship with these cells. Three additional observations strengthen this conclusion. (i) All of the guidepost cells serve as contact points at which later arising neurons join the pioneer pathway (Figs. 3C and 4, C and E). (ii) A hypothesis of guidance by a gradient on the scale of the whole limb is made unlikely by the sharp turns in the pathway, which indicate the presence of local signals. (iii) A hypothesis of guidance by growth along a morphological (not molecular) feature of the substrate is made unattractive by transmission (Fig. 4D) and scanning (*18*) electron microscopic examination of the pathway taken by the pioneers; no suitably oriented discontinuities are apparent. If this guidepost hypothesis is valid, it may be that all neurons in this system can serve as pathway guideposts as well as functional neurons and that it is only an unusual situation in early embryogenesis, the delay between guidepost cell birth and axonogenesis, which has allowed these dual roles to be observed independently.

Filopodial Exploration

Since the guidepost cells are not contiguous, a mechanism for locating successive cells would have to be available to the pioneer growth cones. In vitro studies have emphasized the role of filopodia in locating targets (*1*), and it is possible that the spaces between guidepost cells could be bridged by random (at least nondirected) filopodial exploration. Profuse filopodia are characteristic of grasshopper embryonic neurons (*9, 20*), and this is also the case for the first pioneers. Intracellular dye injections reveal numerous filopodia extending from the cell body, axon, and particularly in advance of the growth cone (*18*) (Fig. 2A). We have not been able to observe the tips of filopodia in living embryos, but the filopodial bases are visible under interference contrast optics. Under such



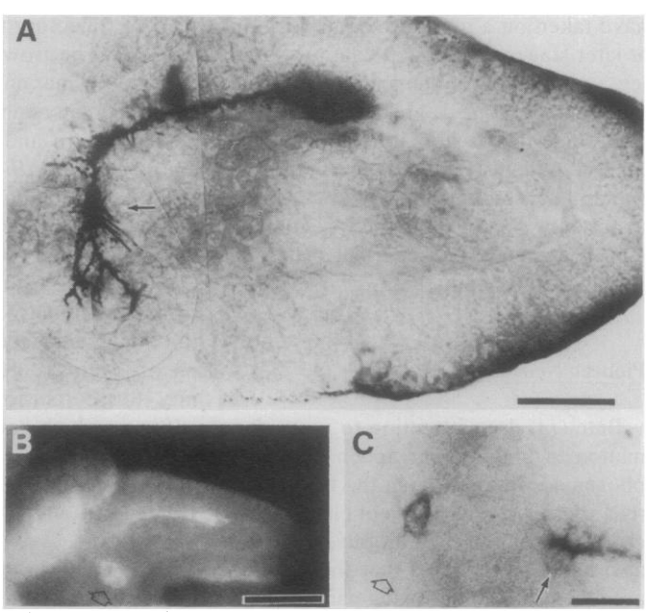


Fig. 1 (left). Embryogenesis of grasshopper limbs. (A) An embryo at 40 percent of embryonic development (scanning electron micrograph). The arrow indicates metathoracic leg. (B) The metathoracic leg at 55 percent of development (photomicrograph). The form of the adult leg has been established with segmentation, intrinsic musculature, cuticular apodeme (for muscle insertion), and nerves (*C*, coxa; *T*, trochanter; *F*, femur; *Ti*, tibia; *Ta*, tarsus). (C) Tip of the metathoracic leg at 30 percent of embryogenesis (Nomarski photomicrograph). An epithelium surrounds the limb, with mesodermal cells visible in the lumen. The arrow indicates the first pair of pioneer neurons, after it has emerged from the epithelium but before axonogenesis. Fig. 2 (right). Pioneer neurons and guidepost cells. (A) One neuron from the first pioneer pair during growth cone navigation at 33 percent of development (silver-intensified intracellular cobalt injection). The growth cone (arrow) has made the first turn and is traversing the ventral epithelium. The profuse and lengthy filopodia at this stage span the limb lumen. Scale, 50 μm. (B) First pioneer pair and second pioneer pair (arrow; cells CT1 in Fig. 3) at 33 percent of embryogenesis. The limb has been stained with an antibody which binds to neurons (*5*). No axons are present in the distal limb other than those of the first pioneer pair. Scale, 100 μm. (C) A "guidepost" cell (unfilled arrow; F2 in Fig. 3) displaying on its surface the bound antibody to peroxidase (*5*). The cell has no axon, and no surrounding cells are binding antibody. The approaching growth cone (solid arrow) is from the first pioneer pair. Scale, 25 μm.

optics, it can be seen that filopodia are continually extended in different directions. Measurement of filopodia of fixed, dye-filled cells show that they are often as long as 50 μm, and some are longer than 75 μm. These lengths are more than adequate to cross the gaps between guidepost cells. In Fig. 2A, a pioneer has been dye-filled just at the point when it should be reaching the third (CT1) guidepost. The filopodia at this time span the entire width of the lumen. In that guidepost cells F1 and F2 are both situated along the initial, straight segment of the pioneer axons, it is evident that the spaces between the cells could be readily spanned by filopodia.

If guidepost cells are located by random filopodial exploration, and if no additional guidance information is present, then filopodia should not be initially oriented in the direction of the next cell in the pathway. Analysis of growth cone morphology does not provide easily interpretable information on this point because a growth cone is itself a polarized structure, with an axon at the trailing margin and filopodia projecting from the leading edge. Thus, once the pioneer has initiated axonogenesis, many filopodia are inevitably oriented along the pathway. Consequently, a more revealing test is to examine the disposition of filopodia before axonogenesis. The pioneers are not suitable for this test since at this stage in their differentiation they lie in the tip of a cone (topologically). Instead, we have examined this stage of differentiation in a different afferent neuron, cell F1. This cell lies underneath the pioneer axons and will eventually project its axon directly along the pioneers (Fig. 4B). Before axonogenesis, cell F1 generates a dense corona of filopodia; these filopodia are characteristically radial in disposition and are not oriented in the direction which the growth cone will eventually take (*18*) (Fig. 4A). This indicates that extrinsic directional cues which initially direct filopodial extension are not present.

Pioneer Pathway:

Navigation and Displacement

The axons of the pioneers will eventually provide a continuous connection, which will be followed by other neurons, between the pioneer cell bodies, located at a particular site in the tibia (Fig. 3C), and the CNS. We have discussed above how that connection is established by navigation of the pioneer axonal growth cones. Ultimately, much more of the path provided by the pioneer axons is generated by elongation of the axon after connection to the CNS. This growth is necessitated by displacement of the cell body away from the CNS. When the pioneers first emerge from the epithelium, they are less than 200 μm from the CNS (*16*). However, the intervening limb tissue continues to grow rapidly, confronting the pioneers with a receding target. By the time the connection to the CNS is established, the cell body to CNS distance is greater than 300 μm; this is the maximum length contributed to the axon by growth cone navigation. By the 55 percent stage (Fig. 1B), the pioneer axons are over 1000 μm long and, in adult grasshoppers, the corresponding distance is more than 35,000 μm. Thus, most of the path provided by the pioneer axons, particularly for later arising sensory neurons, is generated by cell body displacement with concomitant axonal growth. In other words, an important aspect of the morphogenetic mechanism for generating a long-distance pathway in this system is that the first connections between the periphery and CNS are made when the distances involved are very short, and then the axons of the pioneer cells mediating the pathway elongate over great distances, all the while remaining connected to their target.

Pioneers Found Nerve Trunks

The propensity of neuronal growth cones to grow along oriented substrates, an example of "contact guidance," is a common property of developing nervous systems and is well known in insects (*3, 4*). Pioneer axons provide a contact guidance pathway for the axons of subsequently generated neurons. In the cercal appendages of crickets (*14*) and in grasshopper antennae (*21*), electron micrographs have been made of the nerves throughout embryogenesis starting with the first appearance of pioneer axons. These show a continual fasciculation of axons onto the pioneers, eventually forming thick nerve trunks. At the time of hatching, these nerve trunks in the antenna contain more than 6000 axons. Therefore, pioneers are the founder axons of the nerve branches seen in the mature peripheral nervous system.

The first pair of pioneers in the grasshopper leg found a nerve branch that is well characterized in adults, branch 5B1 (*22*) (Fig. 3, A and C). Distally, this branch is easily identified because it receives the axons of two prominent sensory organs, the femoral chordotonal organ (FCO), and the subgenual organ (SGO) (Fig. 3A). In the embryo, sensory neurons of the FCO and SGO begin to appear at the 35 and 40 percent stages,

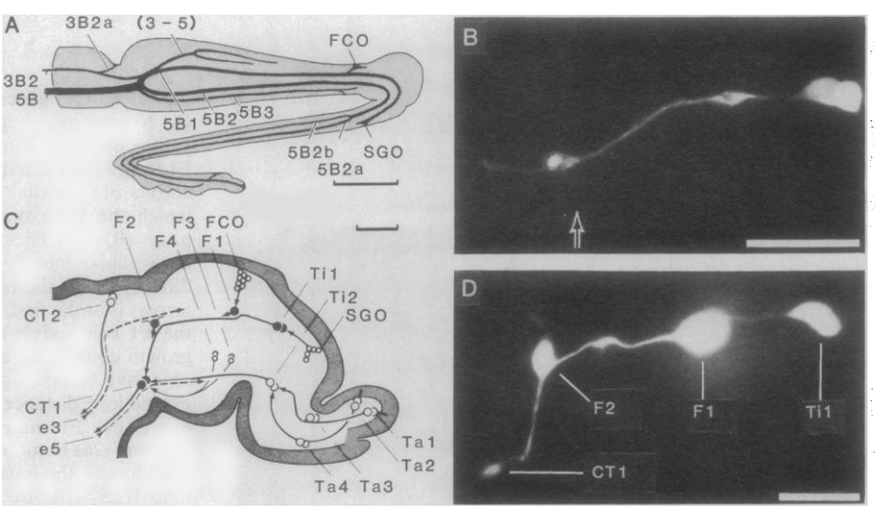


Fig. 3. Nerve pathways. (A) Principal nerve branches in the adult leg (*2*). Note branch 5B1, which is established by the first pioneer pair, and the site of the femoral chordotonal organ (FCO) and the subgenual organ (SGO). Scale, 5 mm. (B) The second pioneer pair (CT1) to appear in the leg with antibody staining (intracellular injection of Lucifer yellow dye). The cells are the last "guidepost" cells for the first pioneers (Fig. 2B) and make the first connection to the CNS (*19*), except in the metathorax (*13*). Arrow indicates the border of the CNS. Scale, 50 μm. (C) Diagram of a 45 percent leg showing the cells that establish the major peripheral nerve branches (*33*). Solid lines indicate afferent neurons, and dashed lines indicate efferent neurons. Filled cell bodies are those of the first pioneers and the "guidepost" cells along their route (D). Where afferent and efferent routes overlap, the thicker lines indicate which cell pioneers the region first. Scale, 100 μm. (D). Selective cell-to-cell junctions between the first pioneers and the "guidepost" cells. The fluorescent dye Lucifer yellow has been injected intracellularly into cell F1 and has migrated into F2, Ti1, and CT1, demonstrating dye-coupling junctions among these cells. The dye has not appeared in any other cells. Scale, 50 μm.

respectively, and project their axons onto cell F1 and onto the pioneers (*8, 18*) (Figs. 3C and 4, C and E). The axons of these and many other afferent neurons grow along the pioneers toward the CNS, forming branch 5B1.

The other branches of the peripheral nervous system in the leg are established by other neurons. These cells, and the branches to which they give rise, are described in Fig. 3C. Two important features of these pioneers should be noted. (i) The cells are identifiable. Particular cells or cell pairs always arise at the same time and at the same location in the limb. There is a set constellation of pioneers that provides the scaffolding for the nerves (the only exception observed is that occasionally only a single cell appears instead of a pair). (ii) Some routes are pioneered by efferent axons. Efferents may grow for some distance along afferent pioneers and then diverge, may form routes completely independently, or may form a route by the collision of afferent and efferent growth cones (Fig. 3C).

The formation of the afferent peripheral nervous system is a sequential process which is not completed until adulthood. Essentially, a convergent array of cells is established, with newly generated neurons added at the perimeter throughout embryonic and postembryonic development. The pioneers provide the initial core of this array. Among the pioneers, those in the most proximal portion of the limb (cells CT1) (Fig. 3C) generally connect to the CNS first; pioneers in the tibia and femur project onto them, and later arising pioneers (for example, Ta1 and Ta2) project onto tibial pioneers (Ti2). Consequently, if the guidepost hypothesis is valid, the more distal cells connected into the array serve as guideposts for newly generated neurons further in the periphery which are initiating axonogenesis.

Development Without Pioneers

The developmental role of pioneers can be assessed by experimental manipulation. The first step in this analysis was made by Edwards *et al.* (*23*), who examined nerve morphogenesis in the cercal appendages of crickets. These appendages have distally situated pioneers which normally found two nerves. With a laser directed through the eggshell, the tip of the cercus was lesioned before the time of pioneer axonogenesis (*23*), and the eggs were then incubated until substantial cercal nerves should have formed. Electron micrograph sections made across the cercus showed that, in the typical case, several nerve bundles were present rather than the usual two. If the pertinent damage was confined to the pioneers, this experiment indicates that, at least under some circumstances, absence of the pioneers results in abnormal nerve morphogenesis.

Does this mean that pioneer neurons have a pathfinding capacity not shared by other neurons? In the grasshopper leg, it has been possible to address this question more specifically because the pioneers can be selectively deleted and because the landscape in which pioneer growth cones navigate is known in terms of identified cells.

To determine whether nonpioneer neurons can follow the path taken by the pioneers, we studied a neuron that normally does not have to pathfind at all. Cell F1 is in contact with the pioneers' axons when it undergoes axonogenesis and grows along them (Fig. 4B). To ascertain whether it could find the normal path to the CNS in the absence of the pioneers, we removed embryos from the egg before the 30 percent stage, impaled the pioneers before axonogenesis with microelectrodes, filled the cells with Lucifer yellow dye, and selectively destroyed them by photoinactivation (*24*). The embryos were then rejoined with a cluster of yolk cells and cultured in hanging drops (*25*). The morphology of cell F1 in experimental limbs and in contralateral control limbs, was examined by intracellular dye injection or by staining (a limb) with labeled antibody

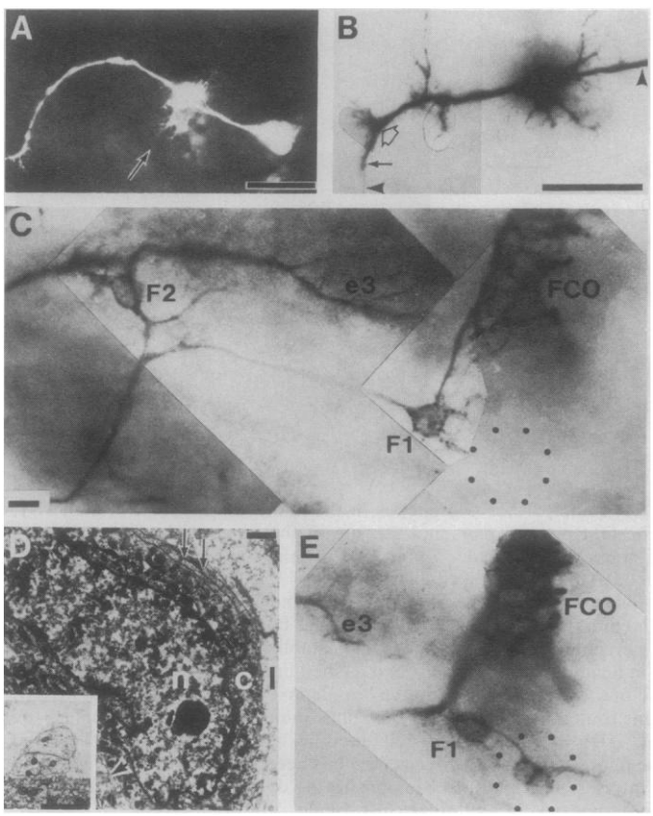


Fig. 4. Morphogenesis of cell F1 with and without pioneer neurons. (A) Normal morphogenesis of F1 at 34 percent stage (double Lucifer yellow dye injection of F1 and Ti1 pioneers). Prior to axonogenesis, F1 (arrow) is located directly on the pioneer axons and has a radiating corona of filopodia. These filopodia are not oriented in the direction which the F1 axonal growth cone will eventually take. Scale, 50 μm. (B) Cell F1 shortly after the onset of axonogenesis (double intracellular injection of Lucifer yellow dye) The axon is growing directly along the pioneers (arrowheads); the growth cone (unfilled arrow) is at the first turn, where cell F2 is located (about 50 μm from the F1 cell body); a characteristic thick leading filament from the growth cone (solid arrow) is making the turn along the pioneer; the extensive filopodia remaining at the cell body and along the F1 axon extend long distances from the path. Scale, 50 μm. (C) Morphogenesis of cell F1 and others after experimental removal of the pioneer neurons (*26*) (antibody stain). Dotted circle indicates the site corresponding to the location of the pioneers in the contralateral control limb (Fig. 4E). In the absence of pioneers (Ti1), a normal axon trajectory has been established by cell F1 (and also by F2, FCO, e3, and others). Scale, 25 μm. (D) Electron micrograph showing direct membrane apposition between the Ti1 pioneers (longitudinal section; arrows) and cell F1 (*n*, nucleus; *c*, cytoplasm; *l*, limb lumen, arrowhead, boundary between cell F1 and limb epithelium). (Inset) Same pioneer axon pair in cross section. Scale, 1 μm. (E) Neuron morphogenesis in the contralateral, control limb of the operated, cultured embryo illustrated in (C) (antibody stain). Dotted circle surrounds the Ti1 pioneer neuron pair. As in the normal case, the pioneers project onto cell F1, as do the axons of the FCO; nerve 3 efferents (e3) are approaching in the normal location, and the axons of SGO (lower right corner) can be seen projecting onto the pioneers. Scale, 1 μm.

(*26*). Cell F1 projected its axons along the normal pathway both in the control limbs and in the experimental limbs (Fig. 4, C and E). Therefore, neurons other than the pioneers are able to follow the pioneer pathway.

We suggest that what is being revealed by both the cricket and grasshopper experiments is a basic pathfinding capability common to all neurons in this system. Sensory neurons arise continually in the epithelium until the animal reaches adulthood. Almost every cell must "pioneer" at least a short route. All may accomplish this by the same mechanism: undirected filopodial exploration, contact with an acceptable "guidepost" cell (which may include every neuron), and growth along the axon of that cell by contact guidance. This behavior would generate the normal pattern of converging nerves. In our experiment, after the pioneers were removed, the next nearest cell within filopodial reach for cell F1 would be cell F2, and growing along F2 would establish a normal route. In the cricket experiment, after the tip pioneers were removed the next nearest cells would probably be neurons located at the base of the cercus (*27*), and newly arising neurons at the tip might well grow to them by several routes as observed. In a well-known experiment, Wigglesworth (*3*) showed that under extreme circumstances, sensory neuron growth cones will even grow back in loops onto their own axons, forming circular bundles. The routes taken by axons in each of these experimental situations are consistent with the hypothesis that what underlies abnormal routing is not differences in pathfinding capability but rather the disposition of acceptable guidepost cells in the cellular landscape.

In the absence of exclusive pathfinding capability, how should the morphogenetic role of pioneers be viewed? If guidepost cells are being located by filopodial exploration, then connections must be made before the distances between cells exceed the span of filopodia. Because of the rate of limb growth, it may be critical that neurons be generated and connected almost as soon as the limb bud evaginates. The pioneers do make such connections. In other words, rather than our considering that the purpose of pioneers is to found particular nerves, it may be more appropriate to view these nerves as a consequence of having to connect neurons to the CNS early in limb development.

Much information on pattern formation in morphogenetic fields of cells has been provided by study of the insect epidermis (*28*). The work of Anderson and Bacon (*29*), of Ghysen (*30*), and of Palka *et al.* (*31*), and particularly the detailed topological studies of Murphey (*32*) have shown that the axonal arborizations made by sensory neurons at their target sites in the CNS are determined by their position within the growing epithelium. If the guidepost system does underlie pathfinding, then the regularity of structure of the peripheral nervous system within the limb is caused by the regularity with which neurons are generated at certain times and places in the developing leg. In every case examined [Ti1 (*16*), Ta1, and CT2 (*18*)], the afferent pioneers have originated within the epithelium. Under these circumstances, the question of how long-distance pathways are established in this system ultimately becomes a question of patterned cell determination in a morphogenetic field.

Conclusions

The first neurons to appear in the developing grasshopper metathoracic leg arise from the epithelium at the limb tip and project axonal growth cones toward the CNS. These are the first "pioneer" neurons. Growth cones from these pioneers grow from cell to cell along a chain of "guidepost" cells. Guidepost cells display cell-surface molecules which distinguish them from surrounding cells, and pioneer growth cones selectively establish junctions with them. Guidepost cells may constitute the pathway which is followed by these pioneers to the CNS. Intervals between guidepost cells can be spanned by filopodia from pioneer growth cones. After the pioneer axons are connected to the CNS by growth cone navigation, the cell bodies are secondarily displaced to relatively great distances along the limb by the growth of intervening tissue. Axons of later arising neurons fasciculate on the pioneers, so that the pioneers found the characteristic pattern of nerve branches of adults. A specific set of pioneers (both afferent and efferent) establishes the scaffolding of the peripheral nervous system. Morphogenesis of identified neurons after selective deletion of pioneers shows that nonpioneers can navigate the pioneer route, indicating that it is the spatial and temporal disposition of neurons, rather than inherent pathfinding capacity, which is important for establishment of normal nerve trunks. The regularity of peripheral nerves in this system appears to be due to the precision with which cells become determined as neurons in the developing limb epithelium.

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25. The medium consisted of NaCl, 150 m*M*; KCl, 10 mM; $CaCl_2 \cdot 2H_2O$, 4 m*M*; $MgSO_4 \cdot 7H_2O$, 2 mM; Tes, 5 mM; sucrose, 125 mM; phenol red, 30 mg/liter; Gibco antibiotic-antimycotic mixture, 1 ml per 100 ml of solution; *p*H 7.2; incubation at 30°C.
26. In nine experiments where F1 or F2 initiated axonogenesis in the absence of the Ti1 pioneers, all aspects of axonal pathfinding which were examined were normal in eight cases; in five embryos stained with antibody after the 40 percent stage, F1 and F2 projections were normal; in three embryos where F2 was filled intracellularly with Lucifer yellow dye at 35 to 40 percent stages, an axon was projected in the normal direction in each case; in one case stained with antibody and subsequently filled with Lucifer yellow, F2 projected in the normal direction but F1 projected a short axon about one cell diameter in an abnormal direction (posteriorly).
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33. CT1, F1, F2, and Ti1 (see text). *Ti2*: pioneer(s), appearing by 40 percent, connecting to CT1, founding distal 5B2. *Ta1*: pioneer(s), appearing by 38 percent, maintaining epithelial dendrite, connecting to Ti2, founding distal 5B2a. *Ta2*: pioneer(s), appearing by 39 percent, maintaining epithelial dendrite, connecting to Ti2, founding distal 5B2b. *Ta3*, *Ta4*: cell pairs, appearing by 40 percent, connecting to Ti2. *F3*, *F4*; pioneers, appearing by 35 percent, connecting to CT1 or 5B. *CT2*: pioneer(s) emerging from epithelium by 35 percent, contacting CNS via e3, founding distal 3b2a. *e3*: efferent pioneer(s), emerging by 35 percent, founding proximal 3B2a, contacting CT2 axons. *e5*: efferent pioneer(s), emerging by 34 percent, founding proximal 5B2. Abbreviations: SGO, subgenual organ; FCO, femoral chordotonal organ.
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Breast-Feeding Patterns in Low-Income Countries

Barry M. Popkin, Richard E. Bilsborrow, John S. Akin

Summary. Breast-feeding is important to infant nutrition, morbidity, and mortality, and to postpartum amenorrhea (hence to birth intervals). Evidence on breast-feeding patterns in low-income countries from nationally representative World Fertility Surveys and secondary sources shows that in all but a few such countries most children are breast-fed for at least a few months. The limited evidence available on trends seems to indicate a decline in the duration of breast-feeding, but in most of Asia and Africa breast-feeding is almost universal during at least the first 6 months. Earlier weaning is common in Latin America.

Considerable research has been conducted in developing countries on the potential benefits of breast-feeding, particularly its effects on infant health and postpartum amenorrhea, but information about actual breast-feeding practices is scattered and incomplete. Here we examine the available information, including new data derived from national surveys in 17 low-income countries.

Breast-feeding has important social and economic consequences in low-income countries. It is a dependable means of providing infants with a nutritious and easily absorbed food and with immunological protection against certain diseases. In addition, by prolonging postpartum amenorrhea, it increases the interval between births. Any decline in breast-feeding in low-income countries is therefore a matter for concern (*1–4*).

Breast-Feeding Patterns

Today there is wide variation in the extent and duration of breast-feeding. Vahlquist (*5*), Knodel (*4*), and Hirschman and Butler (*6*) cite evidence of a rapid decline of breast-feeding in Europe and the United States between the 1930's and the 1960's. Somewhat of a resurgence seems to have begun in those parts of the world in the 1970's (*7*), but modernization is thought to be leading to a tendency towards earlier weaning and discontinuation of breast-feeding in low-income countries (*8–10*), particularly in their urban, periurban, and more modern rural areas. Only scattered statistical evidence has been available, however.

Moreover, it has been extremely difficult to document trends over time even when estimates from more than one time are available. This is because existing studies focus on entirely different populations, use different and sometimes inappropriate definitions of breast-feeding, and are based on nonrepresentative samples, usually from hospitals, clinics, or single communities. For example, a study conducted in a low-income area of Bogotá in 1968 and 1977 was reported to show a 2-month decline in the mean duration of breast-feeding (*11*); however, the 1968 data came from a sample of health center users and the 1977 data from a sample of the general population. A frequently cited study estimated a 20 percent decline in the Philippines between 1958 and 1968 (*8, 12*), but the data came from entirely different and noncomparable populations—the 1958 data from metropolitan Manila, the 1968 data from one area each in Luzon, Mindanao, and the Visayas—and for neither was a probability sample used. These studies and a larger number of small, single-round surveys and participant-observation studies have led to the widespread impression that the basic factors regarding the worldwide trend in breast-feeding are far better established than they really are (*3, 8*).

Some nationally representative surveys provide useful data for longitudinal or trend analyses for four countries in Asia and one in Latin America. In peninsular Malaysia there seems to have been a large decline in breast-feeding between 1950 and 1970–74. On the basis of retrospective life-history data from a nationally representative sample of 1262 women under age 50, Butz and DaVanzo estimated that the proportion of infants breast-fed declined from about 94 to 75 percent (*9*). Using multivariate techniques they found a significant inverse association between a child's year of birth and its probability of being breast-fed (*13, 14*), and a 2.9-month reduction in the mean duration of breast-feeding.

Breast-feeding probably declined also in Thailand between 1969–70 and 1979, according to data from a series of national household surveys which seem approximately comparable over time (*15*). The proportion of children who were never breast-fed was very low and changed little between surveys, but the mean duration declined by about 5 months (from 22.4 to 17.4 months for rural children and from 12.9 to 8.4 for urban). Longitudinal analysis in Korea based on the 1974 Korean National Fertility Survey revealed that from 1950 to 1970 the proportions of children breast-

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