FORMATION OF THE RECEPTIVE FIELDS OF LEECH MECHANOSENSORY NEURONS DURING EMBRYONIC DEVELOPMENT[1]

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Abstract

We have charted the embryonic development of the epidermal receptive fields of the P_V and P_D mechanosensory neurons present in each segmental ganglion of the ventral nerve cord of the glossiphoniid leech *Haementeria ghilianii*. The receptive field of either of these neurons is established by several peripheral axons, each of which grows into its own skin territory, so that the receptive field is subdivided into a quiltwork of contiguous, mutually exclusive subfields of various sizes. The largest subfield develops first from the primary peripheral axon of the P_D or P_V neuron. The P_D and P_V primary axons grow directly to their respective territories and begin to innervate them at approximately the same time (see also Kuwada, J. Y., and A. P. Kramer (1983) J. Neurosci. *3:* 2098-2111). The smaller secondary and minor subfields develop later from the secondary and intersegmental peripheral axons, respectively. These axons, too, grow directly to their appropriate skin territories. The arborizations of the peripheral axons expand until the adult receptive field pattern is established late in embryogenesis; they do not appear to initially overgrow and later trim down to the normal boundaries of the adult receptive field. The receptive field of the P_V neuron develops from a stereotyped skeletal branching pattern of the peripheral axons that is elaborated in a regular way to form a grid-like pattern that matches the arrangement of muscle fibers in the body wall. Thus, this branching pattern may be the result of axon growth along prespecified pathways, perhaps delineated by the muscles. Throughout embryonic development, as in the adult leech, axon branches of neighboring homologous P neurons overlap considerably, but separate axon branches of the same neuron are virtually non-overlapping. These observations suggest that developing branches of a neuron are excluded from territory occupied by other branches of the same cell, in contrast to branches belonging to different neurons which can occupy the same territory.

The mechanosensory neurons in the ventral nerve cord of the glossiphoniid leech *Haementeria ghilianii* are particularly suitable for examining how neuronal processes find a specific set of target cells during development. Each mechanosensory neuron projects axons from the

CNS into a particular territory of skin, where endings of the axons form specialized mechanoreceptors (Blackshaw, 1981a, b). The territory of skin innervated by these receptors is the neuron's receptive field. In each segmental ganglion of the *Haementeria* CNS, there are two bilateral pairs of mechanosensory P (for "pressure") neurons that respond to moderate deformation of the skin, designated as P_V and P_D (Kramer and Goldman, 1981). They are homologous to the P_V and P_D neurons first identified by Nicholls and Baylor (1968) in the hirudinid leech *Hirudo medicinalis*. These neurons have distinct peripheral axon branching patterns and receptive fields in the ipsilateral segmental skin; the field of P_V occupies the ventral skin and that of P_D occupies the dorsal as well as much of the ventral skin (Kramer and Goldman, 1981). The embryonic development of these mechanosensory neurons can be followed from the time of the initial outgrowth of their axons. Their initial

growth is directed along specific pathways that lead to their appropriate receptive field territories (Kuwada and Kramer, 1983).

In the work presented here, the embryonic growth pattern of the peripheral axons of the P_V and P_D mechanosensory neurons has been followed anatomically to determine how these neurons construct their respective receptive fields. Do they initially innervate only the appropriate territory of skin or do they also innervate inappropriate skin territory from which their axons later withdraw? Another question we addressed arises from the following situation. In the adult leech, the receptive field of each mechanosensory neuron is divided into subfields, each of which is innervated by a different peripheral axon. These subfields occupy contiguous but non-overlapping areas of skin. However, these same subfields overlap extensively with the receptive fields of neighboring homologous mechanosensory neurons (Nicholls and Baylor, 1968; Yau, 1976; Kramer and Goldman, 1981). Is it possible, then, that receptive subfields are non-overlapping in the adult because, during development, the different peripheral axons growing from the same neuron avoid each other or compete for a place on their common target? If this were the case, and it appears to be, axons belonging to the same neuron would have to be distinguishable from axons belonging to the homologous neurons with which they can share the same area of skin.

Materials and Methods

The materials and methods used in the experiments of this paper are the same as those described in a previous paper (Kuwada and Kramer, 1983). That paper also presents a general description of the early development of the *H. ghilianii* nervous system and skin territories and an explanation of the system of developmental staging used here. Briefly, stages are indicated by a stage number followed by a fraction in parentheses, which is the fraction of the total duration of the stage that has been completed; the denominator is the stage duration in days.

Results

Innervation of differential skin territories by P_V and P_D neurons during development

How does each of the several peripheral axons extended by the P_V and P_D neurons come to occupy its appropriate skin territory? To address this question we followed the growth of these neurons' axons in the periphery, but before describing the pattern of that growth, it is necessary to review the adult structure of these neurons' receptive fields, which forms the developmental endpoint of our study. The skin of the leech can be divided into 32 segments, each of which is innervated principally by one of the 32 ganglia of the ventral nerve cord (Mann, 1962; Muller et al., 1981). In every midbody segment, the skin of *H. ghilianii* is subdivided into five annuli on the ventral aspect and three annuli on the dorsal aspect, of which the middle one is referred to as the *central annulus*. The receptive fields of the P_V and P_D neurons are centered in the ipsilateral skin of their

own segment and extend into the skin of the adjacent anterior and posterior segments. Also, the field of the P_V neuron extends across the ventral midline and overlaps with the field of the contralateral P_V, and the field of P_D crosses the dorsal midline and overlaps with that of the contralateral P_D. The receptive field of each neuron is divided into non-overlapping *subfields*: a *major* subfield, which is established by axons that project into the segmental skin from the segmental ganglion in which the cell body of the neuron is located, and anterior and posterior *minor* subfields, which are established by intersegmental axons that project from the adjacent anterior and posterior ganglia into the skin of the adjacent segments (Fig. 1). The major subfield is further divided into non-overlapping *primary* and *secondary* subfields (A. P. Kramer and J. R. Goldman, manuscript in preparation), which are established, respectively, by the first (primary) peripheral axon to grow out of the ganglion during development and the subsequent (secondary) axons to grow out (Kuwada and Kramer, 1983). The primary subfield of P_V lies largely in the ipsilateral ventral half of the skin segment, and that of P_D lies in the ipsilateral dorsal half of the skin segment. Both P_V and P_D also have smaller secondary subfields in the ventral skin (Fig. 1).
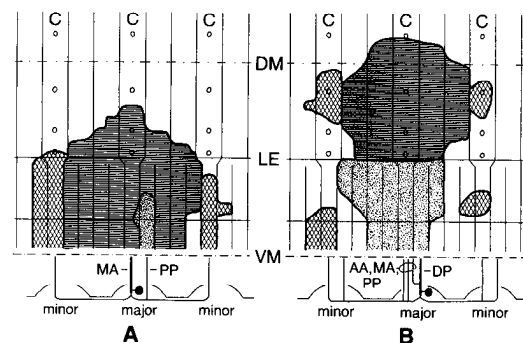

Figure 1. Configuration of averaged receptive field territories of the P mechanosensory neurons in adult *Haementeria* (from A. P. Kramer and J. R. Goldman, manuscript in preparation). Receptive fields are mapped on a schematic representation of three segments of skin on one side of the leech from ventral (*VM*) to dorsal (*DM*) midlines. Although mapped on separate skin representations, the fields of P_V and P_D actually lie in the same skin segments. *LE* is the lateral edge of the body. Leech skin is divided into a series of rings around the body, the *annuli*. Each body segment is divided into three annuli in dorsal skin and five annuli in ventral skin; the *central annulus* (*C*) is the middle annulus of the segment. The complete receptive field of each P neuron is outlined in *bold lines*, except for portions of the field that cross the ventral midline, which have been left out. *Shaded and stippled regions* together constitute the *major* subfield, that is founded by axons originating in the segmental ganglion; *cross-hatched regions* are the *minor* subfields that are founded by axons exiting adjacent ganglia. The *shaded region* of the major subfield is territory innervated by the primary axon of the neuron (schematically shown as a *thick line*) and the *stippled region* is territory innervated by the secondary axons of the neurons (*thin lines*). Anterior is to the *left* in this and other figures. *A*, Field of the P_V neuron. Its primary axon courses in the MA nerve and its secondary axon courses in the PP nerve. *B*, Field of the P_D neuron. Its primary axon courses in the DP nerve and has secondary axons in the MA, PP, and AA nerves.

First we consider how the P_V and P_D neurons form their primary subfields during embryonic development. This was determined by injecting Lucifer Yellow dye into their cell bodies at various stages. These subfields are established by the primary peripheral axons, which are first to grow out of the ganglion. The primary axons of the two neurons have different peripheral targets: the ventral germinal plate for the P_V axon and the dorsal germinal plate for the P_D axon. (The ventral or the dorsal germinal plate is that sheet of embryonic tissue which eventually gives rise to the ventral or dorsal body wall and skin, respectively (Kuwada and Kramer, 1983).) At the very outset of peripheral outgrowth, both P_V and P_D project neurites into the same region of the ventral germinal plate that lies next to the ganglion (Kuwada and Kramer, 1983). However, by the beginning of stage 10 (2 to 3 days after the start of peripheral process outgrowth), P_D and P_V primary axons have grown into different regions of the germinal plate (Fig. 2A). Without forming branches, the primary axon of the P_D neuron takes one circumferential route, which later becomes the dorsal posterior (DP) nerve, directly to the dorsal germinal plate; there it branches extensively. Meanwhile, the primary axon of the P_V neurons grows more slowly along another circumferential route, which later becomes

the medial anterior (MA) nerve. Unlike the P_D primary axon, the P_V primary axon branches extensively in the ventral germinal plate. At this stage, the P_D neuron can still have a few of the initial neurites in the ventral germinal plate (Fig. 2A), remnants of its passage through that region (Kuwada and Kramer, 1983). These remnant neurites later disappear, so that by middle stage 10, the primary P_V and P_D axons occupy completely separate and appropriate territories of the germinal plate (Fig. 2B). At later stages, the primary subfield of the P_V neuron expands into the dorsal germinal plate and by stage 11 begins to overlap there with the primary subfield of the P_D neuron. Thus the primary axons of these two neurons grow initially to different, non-overlapping territories, although they can and do overlap extensively at later stages.

Next we consider how the secondary subfields form. The secondary peripheral axons, which establish these subfields, grow out at a later stage than do primary axons. These secondary axons also grow directly to their appropriate territories, which for both P_V and P_D neurons lie in the ventral germinal plate. For example, at about the time that the remnant neurites of the primary P_D axon are disappearing, the P_D neuron begins to extend a secondary axon into the periphery from its central processes in the ganglion (Fig. 2C). This secondary axon, which grows directly into the MA nerve during early stage 10, branches in the ventral germinal plate and eventually establishes most of the secondary P_D subfield in the ventral skin. In some cases, the P_D neuron extends two more secondary axons, which also grow directly into the ventral germinal plate during stage 10, one in the anterior anterior (AA) nerve and the other in the posterior posterior (PP) nerve (Fig. 2C).

Development of the receptive field of the P_V neuron

To describe the details of the formation of a mechanosensory neuron receptive field, we closely followed the anatomical development of the peripheral axons of the P_V neuron. A series of P_V neurons was filled with Lucifer Yellow dye at successive stages of development, from the time of initial axon outgrowth late in stage 9 through mid-stage 11, by which time the receptive field has achieved its adult configuration. Twenty to 50 neurons were examined at each daily stage through stage 11(5/20), 4 neurons were examined at stage 11(6/20), and 6 neurons were examined at stage 11(9/20).

Formation of the major receptive subfield. Not only does the P_V neuron grow directly to its peripheral target territory, but it arborizes in that territory in a very regular way, as if its peripheral axons are growing along predetermined pathways. The axonal arborization is sufficiently stereotyped in its development that the main branches can be individually named, and even the subsidiary branches grow in the same general pattern from cell to cell. To describe these regularities of axon outgrowth, it is useful to resort to the special nomenclature introduced in the following paragraphs, where the generic names given to types of branches and individual designations given to identified branches are italicized when first mentioned.

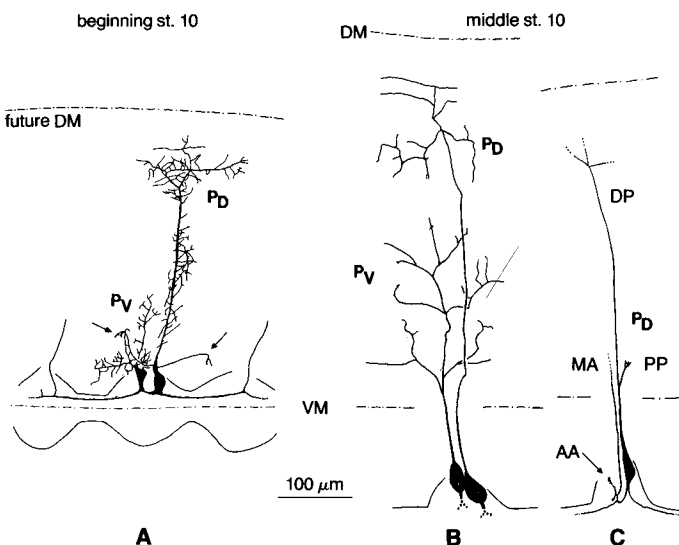The peripheral outgrowth of P_V begins between stages

Figure 2. Tracings made from photographs of whole mounted preparations of P_V and P_D neurons in the same ganglion filled with Lucifer Yellow dye to show comparative growth of their peripheral axons at various developmental stages. *A,* The P_V and P_D neurons at stage 10(0/5). P_V arborizes in the ventral and P_D in the dorsal germinal plate. *Arrows* point to a pair of neurites left in the ventral germinal plate by the P_D neuron. The fine branches on the P_D primary axon between ventral and dorsal fields are lateral filopodia. *B,* The P_V and P_D neurons 2 days later at stage 10(2/5). At this stage all ventral neurites of the P_D neuron have disappeared. *C,* Another P_D neuron also at stage 10(2/5). Secondary axons (*MA* and *PP*) have grown out of the ganglion, or are beginning to grow out (*AA*), into the ventral germinal plate where they will form receptive subfields. At this stage, secondary axons have minimal arborizations in the germinal plate. These secondary axons overlap with the axon branches of the P_V neuron in the same ganglion (not drawn). In this and other tracings, *dotted lines* indicate a process that continues but has not been drawn.

9(2/4) and 9(3/4) with the extension of a single neurite from the lateral pole of the cell body (Fig. 3A). This is the primary axon that will give rise to most of the major subfield. Its *central branch* grows circumferentially, perpendicular to the long axis of the embryo, along that area of the germinal plate from which will arise the central annulus of the segmental skin (see Fig. 1). As this central axon branch elongates, axon branches directed both anteriorly and posteriorly emerge from it perpendicularly at three characteristic circumferential positions. These *first-order longitudinal branches* are designated *B1*, *B2* and *B3*, with the addition of the letters *a* or *p* indicating anterior- or posterior-directed branches, respectively (in accord with the designation of peripheral nerve branches by Ort et al. (1974) and Kretz et al. (1976)). The B1 branches are closest to the ventral midline and the first to emerge, at about stage 9(3/4) (Fig. 3A). The B2 branches emerge next at stage 10(0/5), about halfway between the ventral midline and the future lateral edge of the body wall (Fig. 3B). The B3 branches are the last to emerge, near the future lateral edge (Fig. 3, B and C). Any branches that emerge subsequently from the central axon branch at circumferential positions distal to the B3 branches, although also "first order" from a topological point of view, are considered here to be part of the terminal arborization pattern of the central branch (Fig. 3, C and D). Moreover, some additional, very short longitudinal branches sometimes emerge from the central branch proximal to the B3 branches, particularly between the B1 and B2 branches. Such additional longitudinal branches are most frequently observed during stage 10 (Fig. 3, B to D) and less often during stage 11 (Fig. 3, E and F), suggesting that some of them disappear during stage 11. Furthermore, in less than 1% of the cases examined (an example is in Fig. 7E) did these additional longitudinal branches eventually form the large arborization typical of the three regular first-order longitudinal branches.

It appears, therefore, that the central branch of the primary P_V axon normally begins its peripheral arborization with formation of the B1, B2, and B3 branches. Furthermore, in a given specimen, the B1, B2, and B3 branches of homologous P_V neurons in adjacent segments usually emerge from the central branch at equivalent circumferential positions and grow along the same longitudinal perimeter. Accordingly, some homologous first-order branches growing toward each other from adjacent segments may eventually collide. For example, as seen in Figure 7A, the B1a and B2a branches of P_V of the posterior segment (on the *right*) exactly meet and overlap with the B1p and B2p branches of P_V of the anterior segment (on the *left*). The B3 branches, however, usually do not collide, because most often they grow longitudinally only for a short distance and then make a lateral turn (see following subsection).

As the first-order branches, B1, B2, and B3, grow longitudinally, axon branches emerge from them perpendicularly at characteristic longitudinal positions. These *second-order annular branches* grow circumferentially along the central portions of the future skin annuli, both medially and laterally from the B1 branches, but grow predominantly laterally from the B2 and B3 branches

(Fig. 3, C to E). Whereas growth of the first-order longitudinal branches extends the major field toward the central annuli of adjacent segments, growth of the annular branches increases the density of innervation of the field and spreads it circumferentially. Annular branches cross the future lateral edge and enter the dorsal germinal plate by mid-stage 10 (Fig. 3D), and they cross the ventral midline by early stage 11 (Fig. 3E).

The density of innervation of the field is increased further by the emergence of yet a higher order of branches. Between mid-stage 10 and early stage 11, *third-order longitudinal branches* emerge perpendicularly from the second-order annular branches, closely and evenly spaced (Fig. 3, D and E).

This consistently rectilinear pattern of axon branching and growth probably reflects a corresponding arrangement of some patterning agent or agents in the germinal plate. One of these agents may be the grid of embryonic muscle fibers whose initial development precedes the outgrowth of the peripheral P_V axon (Kramer and Stuart, 1982). The central branch of that axon, as well as its first-, second-, and third-order branches, all grow into the germinal plate between an inner layer of longitudinal muscle fibers and an outer layer of circular muscle fibers. First-order and third-order longitudinal branches are aligned with longitudinal muscle fibers, and the central branch and second-order annular branches are aligned with circular muscle fibers (Fig. 4). Thus the branches form a grid-like pattern (e.g., Fig. 7A) that matches the grid formed by orthogonal longitudinal and circular muscle fibers in the germinal plate.

Formation of sensory endings. The final phase of receptive field formation is the development of sensory endings. These endings are formed by *fourth-order circular branches*, which begin to emerge from the tips of third-order longitudinal branches early in stage 11 (Fig. 3F). These circular branches are directed toward the surface of the germinal plate and appear to grow between the circular muscle layer and the epidermis, forming parallel rows in alignment with the circular muscle fibers (Fig. 3F). Each row consists of a series of circular branches which have emerged from different third-order longitudinal branches and which grow aligned with the same circular muscle fiber (Fig. 9B). Thus the correspondence of axon branching pattern and arrangement of muscle fibers is carried even to the level of the fourth-order branches of the central axon branch. After stage 11(2/20), the circular branches proliferate fine, branched endings into the overlying epidermis. These endings eventually spread into the space between the parallel rows of circular branches, so that the entire epidermis overlying the P_V axon and its branches becomes densely innervated. Axon endings in the skin are irregularly shaped and distributed (Fig. 5A). They are thin (0.2 to 0.4 μm), finger-like processes with occasional swellings at branch points and along their length. Some endings are completely bare along their length (Fig. 5B), whereas others carry very fine processes (Fig. 5C). In several respects, they resemble the skin endings of the mechanosensory T neurons in the adult skin of *H. medicinalis* (Blackshaw, 1981b). Electrophysiological studies not presented here have shown that, by this stage, the axon
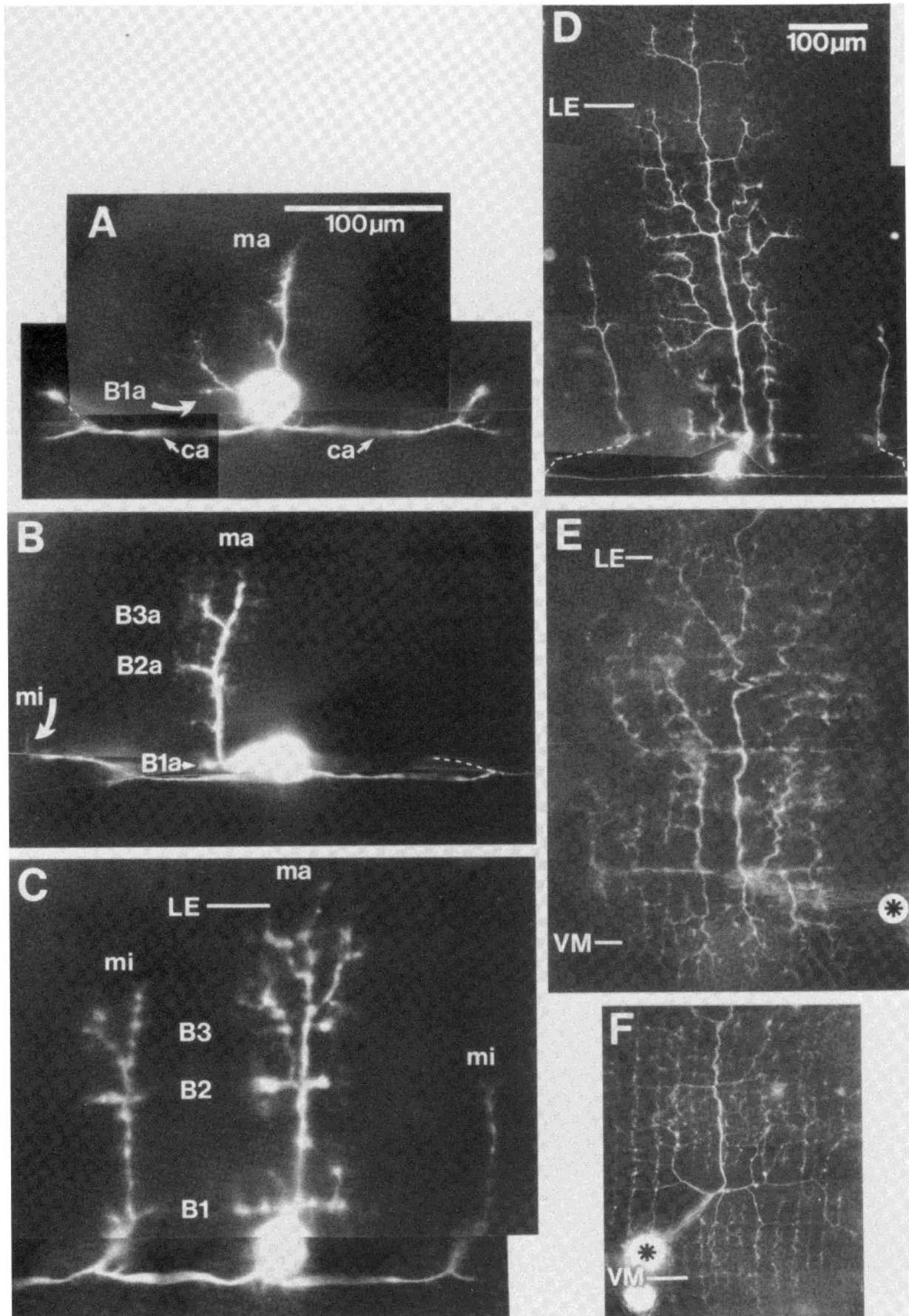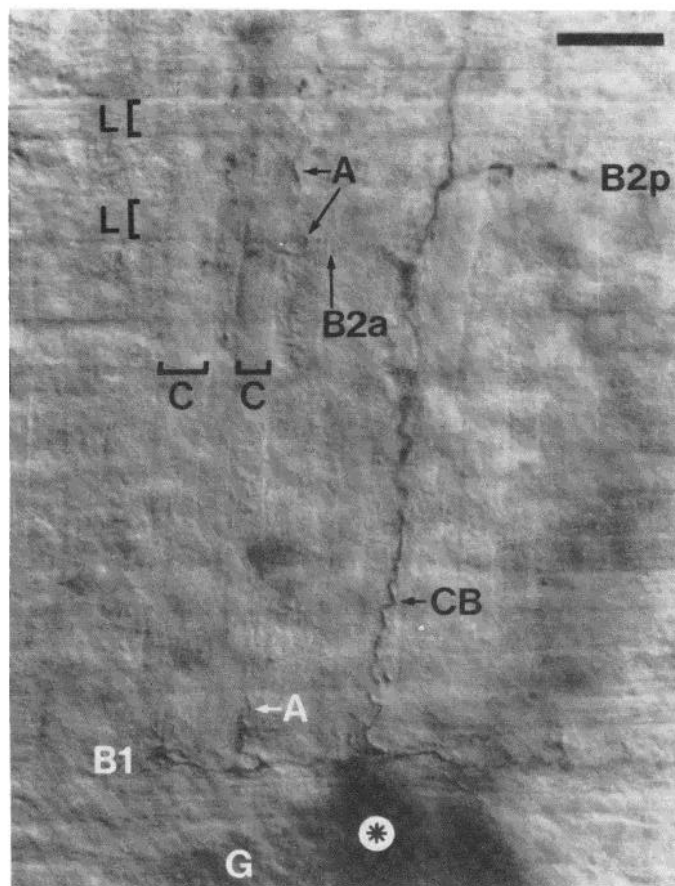
Figure 3

Figure 4. Alignment of peripheral axon branches of a P_V neuron with muscle fibers in the ventral germinal plate of a stage 10(2/5) embryo. Photomicrograph shows the P_V neuron, stained with horseradish peroxidase using a Lucifer Yellow antibody technique (Taghert et al., 1982), and the unstained muscle fibers viewed in whole mount under Nomarski optics. *L* indicates a longitudinal muscle fiber and *C* a circular muscle fiber. There are spaces between adjacent muscle fibers. Oblique muscles are not present at this stage. *G* is the ganglion and the

endings are capable of transducing mechanical stimulation of the skin into action potentials that are conducted to the P_V cell body in the segmental ganglion.

Formation of the minor subfields. The minor subfields, which the P_V neuron establishes in the skin of the adjacent anterior and posterior segments, arise from anteriorly and posteriorly directed branches of the central axon projected from the cell body into the neuropilar region of the ganglion at stage 9(1/4) (Kuwada and Kramer, 1983). These branches enter the connective nerve and, after reaching the next ganglion, turn and leave the CNS to enter the germinal plate of the adjacent segment (Fig. 3, *B* to *D*). The pattern of minor field axon outgrowth and branching is also highly stereotyped and resembles that of major field axon outgrowth. The first-order longitudinal branches projected from the central axon branch of the minor field grow along the same longitudinal perimeter as do those of the major field of the same P_V neuron. Moreover, the minor field axon emerges from its ganglion only after the serially homologous P_V neuron in the same ganglion has already begun to establish its own major field. By filling serially adjacent P_V neurons with Lucifer Yellow dye, it is evident that the central branch and the first-order longitudinal branches of the minor field axon usually follow the paths previously taken by the corresponding branches of the serially homologous major field axon. One difference between minor field and major field axon outgrowth is that in the minor field, the branches usually grow much more slowly and are shorter (Fig. 3, *C* and *D*). However, minor field axons that exit the clitellar or sex (5th and 6th abdominal) ganglia are noticeably thicker than those in other ganglia, and they establish much larger fields (Figs. 3C and 7B).

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asterisk is on the P_V cell body. Branches of peripheral axon are as follows: *CB* is the central branch, B1 and B2 are first-order longitudinal branches, and *A* is the second-order annular branch. *Calibration*, 20 μm.

Figure 3. Successive stages of development of the peripheral axons of the P_V neuron filled with Lucifer Yellow and photographed in whole mounts. All neurons are located in the 10th or 11th midbody ganglia, except the neuron in *C* which is in the 7th ganglion. Note that in these and other pictures of this kind, there is almost always some distortion of the axon branching patterns because the dissected preparation often stretches asymmetrically as it is pinned out. Ganglia, and thus cell bodies, are often displaced longitudinally from the position where the primary axon enters the germinal plate (as in *E* and *F*). Cell bodies are out of focus; hence their size is exaggerated by spread of the fluorescent emission. *Dashed lines* are drawn in to show out-of-focus processes. *A* to *C* are shown at the same magnification and *D* to *F* are shown at half this magnification. *A*, Stage 9(3/4). Primary axon that will establish the major (*ma*) field has entered the germinal plate. A longitudinal, first-order B1a axon branch is just sprouting from the central branch of the primary axon. Central axons (*ca*) extend into adjacent ganglia but do not yet enter the periphery. The finest processes seen in this photograph and in those of *B* to *D* are filopodia. *B*, Stage 10(0/5). B1a, B2a, and B3a branches are present, as is a short longitudinal branch between B1 and B2. A minor (*mi*) field axon has just grown out of the next anterior ganglion (at *arrow*). *C*, Stage 10(1/5). All three first-order longitudinal branches are present and some second-order annular branches have appeared. An annular branch that emerges from the B2a branch has grown into the normal territory of the B3a branch, which is missing. The anterior minor field axons innervate the skin of the 6th (clitellar) segment; their growth is much more advanced at this stage than that of minor field axons in midbody segments. *D*, Stage 10(3/5). Second-order annular branches are well developed and third-order longitudinal branches have begun to emerge from them. The major field axons occupy nearly a full segment width. They have grown across the lateral edge (*LE*). First-order longitudinal branches have just emerged from minor field axons. *E*, Stage 11(0/20). View of the major field axons only. First-order longitudinal branches are longer and new second-order annular branches have grown from the new extensions; there are numerous third-order longitudinal branches. The axons have crossed the ventral midline (*VM*). The cell body is out of view to the *right* of the *asterisk*. *F*, Stage 11(2/20). View of a portion of the major field axons only. Parallel rows of fourth-order circular branches are well developed. An additional cell body in the ganglion had been filled with dye and appears beneath that of the P_V neuron (with *asterisk*); its processes are not visible in the photograph.
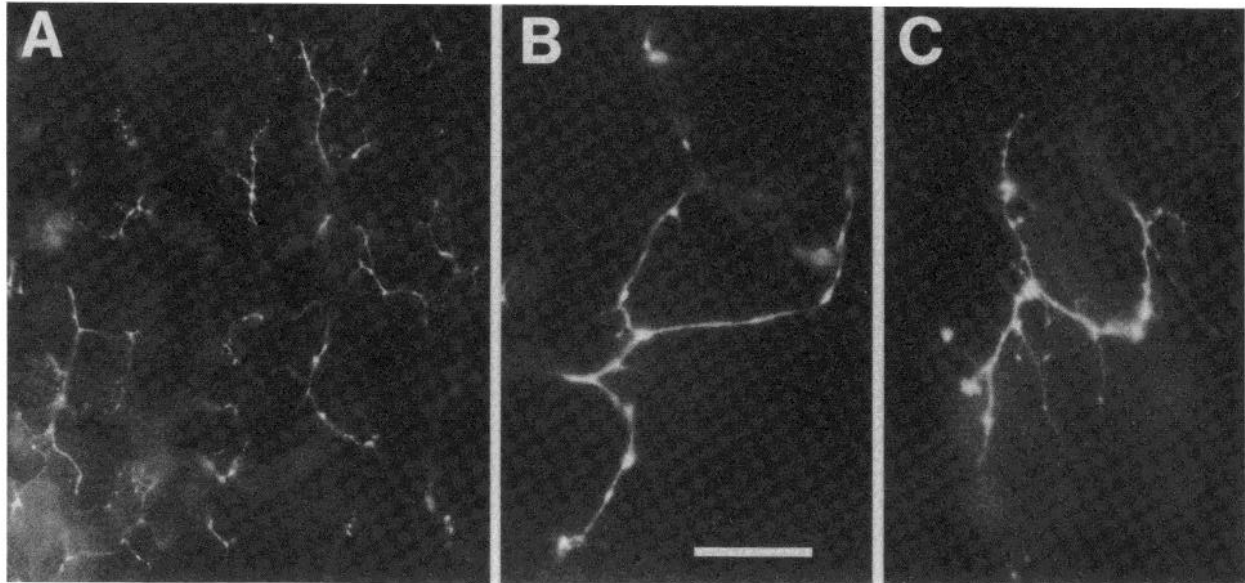
Figure 5. Photographs of skin endings of a P_V neuron filled with Lucifer Yellow in a fixed whole mounted preparation. *A,* Low magnification view in embryo at stage 11(5/20). Endings are near but beneath the surface of the epidermis. *B,* High magnification view in an embryo at stage 11(6/20). *C,* Different ending in same preparation as in *B.* The thinnest processes may be filopodia. *Calibration: A,* 40 μm; *B* and *C,* 10 μm.

Formation of subfield boundaries. A striking feature of the subfields of individual mechanosensory neurons in the adult leech is that they form sharp boundaries, without significant overlap (Nicholls and Baylor, 1968; Yau, 1976; Kramer and Goldman, 1981). In this section, we consider how these sharp subfield boundaries are established during development, focusing attention first on the boundary between the major and minor subfields. As described above, the B1 and B2 first-order longitudinal branches of both the major and minor field axons projected by one P_V neuron grow toward each other along the same longitudinal perimeter and eventually meet. In midbody segments of the embryo, this meeting of major and minor field axons occurs within 1 or 2 days after the start of stage 11. Their branches come very close, and may even touch in a few cases, but they never grow across each other on the same substrate. This mutual avoidance is to be contrasted with the extensive contact and overlap that follows the meeting of axon branches of two serially or contralaterally homologous P_V neurons. Formation of the boundary between the major and minor fields of a P_V neuron is illustrated in Figure 7. At a stage when the major field axons of serially homologous P_V neurons in adjacent midbody segments interdigitate extensively (Fig. 7A), the major and anterior minor field axons of an individual P_V neuron meet but remain separated (Fig. 7B). They remained separated 2 (Fig. 7, *C* and *D*) and 3 (Fig. 7E) days later. Even as late as midstage 11, major and minor field axons of the P_V neuron do not overlap, although a few major and minor field branches, particularly the B1 branches, may appear to contact each other. Most of these apparent contacts are, in fact, cases where major and minor axons are in different levels of tissue and have grown a short way over or under each other at a distance of a few micrometers.

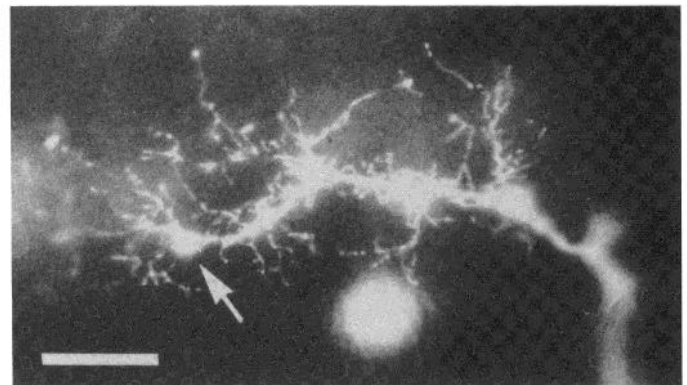It can be inferred, therefore, that the sharp boundary

Figure 6. Photograph of growth cone and filopodia of a B1a peripheral axon branch of a P_V neuron filled with Lucifer Yellow dye at stage 10(1/5). The *arrow* indicates the primary growth cone of this branch. The fluorescent *circular object* beneath the axon is debris. *Calibration,* 20 μm.

between the major and minor subfields is established because the major and minor field axons stop growing toward each other when they meet. Although such an encounter usually ends the longitudinal expansion of the major and minor subfields toward each other, the gaps between these subfields, occasionally observed in adult leeches (Fig. 1), indicate that expansion may sometimes end for other reasons prior to their meeting. The growth-limiting effect of the encounter of major and minor field axons was quantified by measuring the elongation of major and minor field B1 branches, which invariably meet head-on during development (Fig. 7, *B* to *E*). On successive days of development, several P_V neurons were filled with Lucifer Yellow. In each case, the distal ends of the major and minor field B1 branches that were growing toward each other were located and their posi-

tion relative to the two central annuli between which they grew was recorded. As can be seen in Figure 8, by the 5th day after the beginning of outgrowth, the major field B1 branch, growing from its central annulus of origin (C) toward the central annulus of the next segment (C') (Fig. 8, *curve through solid points*), has met and grown past the major field B1 branch of a serially homologous P$_V$ neuron, growing from C' toward C (Fig. 8, *dashed curve*) (see also Fig. 7A). Thereafter, the major field B1 branch approaches but does not grow past the B1 branch of its minor field, growing from C' toward C (Fig. 8, *curve through open points*). The rate of relative growth of the major field B1 branch does not greatly diminish as it approaches the B1 branch of the serially homologous P$_V$ neuron, but that rate falls to zero as the major and minor field B1 branches approach each other. The minor field B1 branch is overlapped by the major field B1 branch of the serially homologous P$_V$ neuron from the very outset of its growth. Its rate of relative growth falls to zero only as it is approached by its own major field B1 branch. Since the embryo continues to lengthen during subsequent development, the major and minor field B1 branches must also increase their absolute length after meeting, but they do not grow past each other.

There are two other instances of self-encounters by axons of the P$_V$ neuron; they also result in a limitation of axon growth. First, the annular branches that grow laterally from the B1 branch eventually encounter the B2 branch, and the annular branches that grow laterally from the B2 branch eventually encounter the B3 branch (at stage 10(2/5); Fig. 9A). Upon such encounters, the annular branches stop short of the first-order longitudinal branches and never grow across them (see also Figs. 3, D and E, and 7, B and C). Sometimes it was observed that an annular branch originating from the B2 branch had grown past the normal lateral position of the B3 branch and thus across the B3's longitudinal line of growth. In these cases, either the B3 branch was missing (e.g., B3a is missing in Fig. 3, C and E) or the B3 branch deviated near the annular branch from its initial line of growth and projected laterally at an oblique angle away from the annular branch (e.g., B3a in Fig. 9A and B3p in Fig. 3D).

The other instance of self-encounter is presented by the fourth-order circular branches that emerge from adjacent third-order longitudinal branches and grow along the same circumferential path. These circular branches are likewise destined for encounter; they grow very close to each other but they do not overlap (Fig. 9B).

It is therefore apparent that whenever axon branches originating from the same P$_V$ cell body encounter each other on the same substrate during development, they do not cross or overlap and rarely even touch.

Formation of the secondary subfield. In addition to the primary subfield, the major field of the P$_V$ neuron occasionally contains a secondary subfield (Fig. 1A). The primary subfield is established by the primary axon growing out of the ganglion along the path of the future MA nerve, whereas the secondary subfield is established by outgrowth of a secondary axon along the PP nerve, from 2 to 7 days after outgrowth of the primary axon

(i.e., during stage 10). In the adult, the primary and secondary subfields are non-overlapping and form sharp boundaries, just as is the case for the major and minor fields of the same neuron (A. P. Kramer and J. R. Goldman, manuscript in preparation). Here too, the non-overlap appears to be attributable to a growth-limiting effect upon self-encounter of axons originating from the same cell body. Thus, from its initial outgrowth through at least early stage 11, the branches of the secondary axon come to occupy only that germinal plate territory which is not yet occupied by branches of the primary axon. For example, when a secondary axon is already present at early stage 10, it occupies that territory which, in its absence, would have been taken by the B1p branch of the primary axon. In this instance, there is no B1p branch, and the branching pattern of the secondary axon actually possesses all general features of the arborization that normally would be derived from the absent B1p branch (Fig. 10A). In other cases, examined later in stage 10, a P$_V$ neuron can have both a secondary axon and the B1p branch of its primary axon. In this instance, the arborization of the secondary axon occupies a rather small region of the body wall lying between the B1p branch arborization and the ventral midline (Fig. 10B).

Filopodia of outgrowing axons. As will be raised under "Discussion," there may be interactions between outgrowing axons which govern receptive field structure. One way the axons could interact is by contact via filopodia, which protrude from the growing axons throughout stages 9 and 10 of development (Figs. 3, A to D, and 9). Filopodia, which extend up to 50 μm, protrude not only from the growth cones that precede each elongating axon branch but also intermittently along its whole length (Fig. 6). During mid- to late stage 10, each axon branch is surrounded by a halo of filopodia which sometimes nearly fills the space between adjacent branches (Fig. 3D). Examination of whole mounted preparations shows that filopodia on different branches of the same neuron appear to contact each other and adjacent axons. However, the adjacent axons themselves rarely touch directly.

Discussion

The highly stereotyped structure of the receptive fields of the P$_V$ and P$_D$ mechanosensory neurons in the skin of the adult leech (Nicholls and Baylor, 1968; Yau, 1976; Kramer and Goldman, 1981; A. P. Kramer and J. R. Goldman, Manuscript in preparation) could arise by either of two rather different developmental processes. Under one alternative, the embryonic neurons would project into, branch within, and establish sensory endings in the germinal plate more or less at random; the regularity of the location and configuration of the overall receptive field and of its subfields would result from subsequent selective elimination of inappropriately placed branches and endings by trophically or functionally mediated "tuning." Under the other alternative, the regularity of the adult field structure would be the result of a directed axonal growth and patterned branching. It is the latter alternative for which we have found evidence, with the stereotyped growth pattern resulting from two developmental processes working in tandem: (1) initial
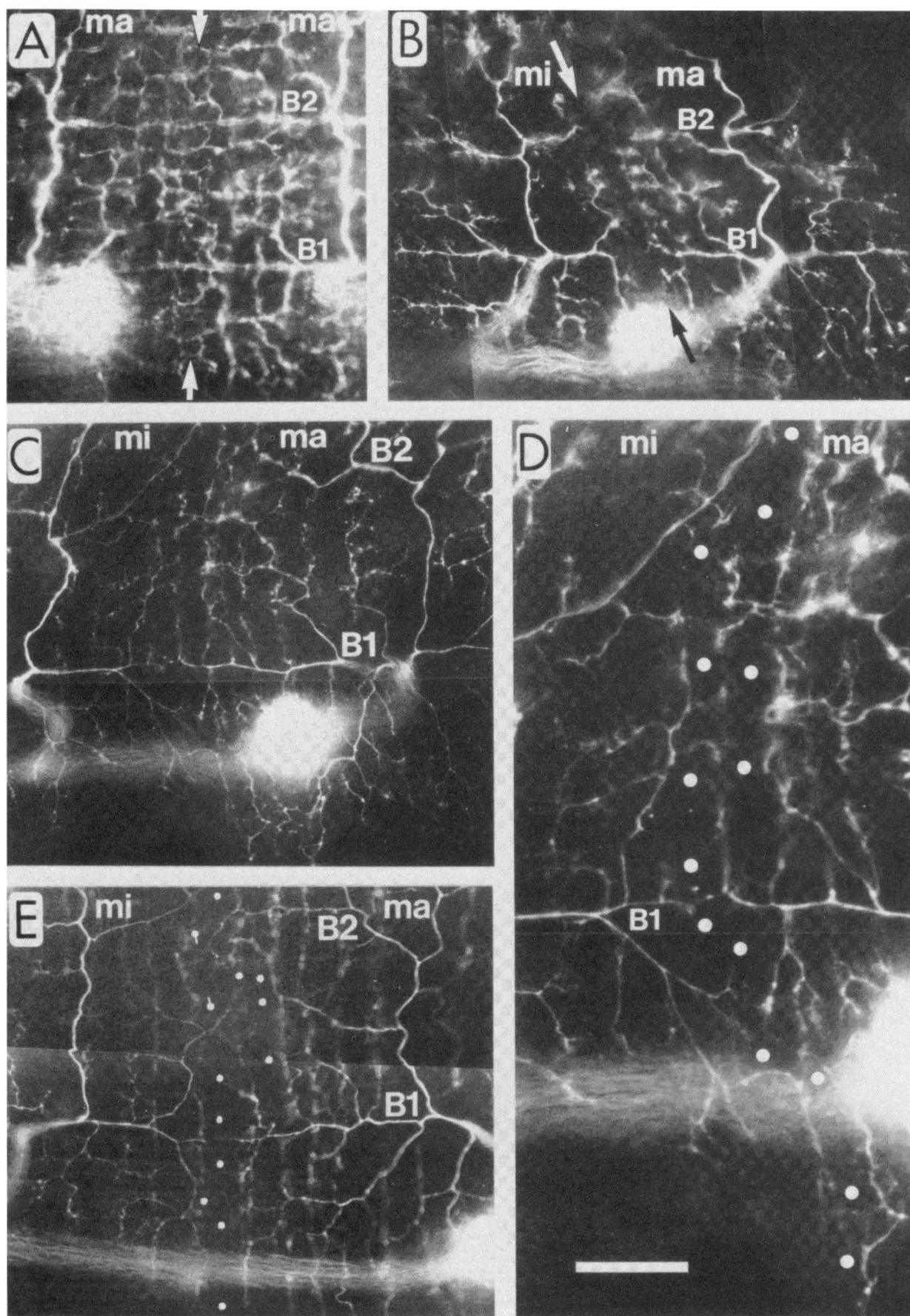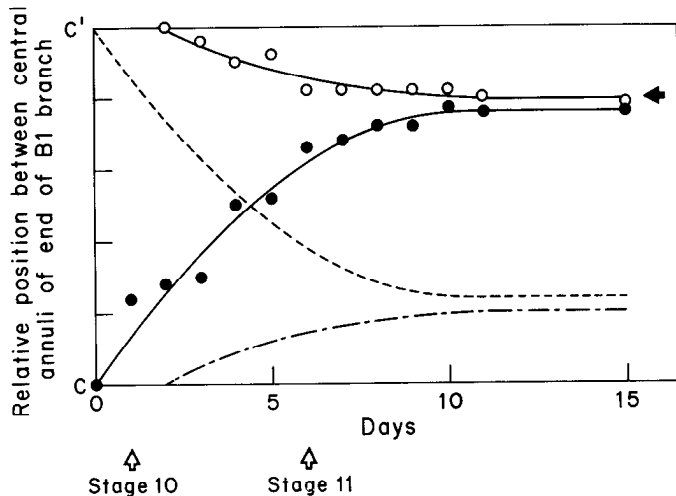
Figure 7

Figure 8. Elongation and approach of the B1 branches of major and minor fields of a P_V neuron. On successive days, 2 to 14 P_V neurons were filled with Lucifer Yellow to determine the position of the distal ends of B1 branches of their major and minor fields. (The origin of this graph lies at the day preceding first outgrowth of the major field B1 branch.) The relative position of the end of the B1 branch between the middle of the central annulus at which it originated and the middle of the central annulus toward which it was growing was measured for major/minor pairs of B1 branches. The *ordinate* shows averages computed for each day. In no specimen had major/minor pairs of B1 branches grown past each other. The B1 branches of major and minor field axons originate at positions C and C′, respectively, and the daily positions of their ends are plotted as *solid* and *open circles*, respectively. Smooth curves were fitted visually to these points. Inversions of these curves are shown also, to represent the presumptive position of the ends of major (*dashed line*) and minor (*dash-dot line*) field B1 branches of the next serially homologous P_V neuron that grow between the same C and C′ annuli. The *arrow* indicates the typical relative position of the boundary between major and minor receptive fields in the skin area innervated by B1 branches in adult leeches.

outgrowth of the several peripheral axons of a neuron preferentially to particular peripheral territories and subsequent growth along particular routes within those territories, and (2) mutual territorial exclusion of these axons. The first process governs the location and general configuration of the receptive field, and the second process governs details of the subfield structure.

Determinants of overall receptive field configuration. Kuffler and Muller (1974), who chanced upon an abnormal adult leech with supernumerary P neurons in a series

of ganglia, observed that the receptive fields of duplicate P neurons were of either a P_V or P_D type. They concluded that each neuron must have an identity with regard to the peripheral territory it will innervate. In agreement with their conclusion, we find that in normal leeches the P_V and P_D neurons exhibit their identity very early in development, by projecting an appropriate set of axons selectively to their appropriate territories (Fig. 2; Kuwada and Kramer, 1983). This is true for all of the peripheral axons of a P neuron, including the primary axons that grow first, the secondary axons that grow later from the segmental ganglion, and the minor field axons that grow out from adjacent ganglia.

Kuffler and Muller (1974) also observed that the receptive fields of duplicate P neurons of the same type can overlap completely. This makes it unlikely that the overall boundary of the P neuron receptive field is governed by interneuronal competition. This inference is confirmed by the present developmental studies. First, the territories occupied by axons of serially and contralaterally homologous P neurons overlap considerably during the middle to late stages of their developmental expansion. Indeed the developing major field of a P_V neuron hardly slows its expansion as it penetrates the major field of an adjacent serially homologous P_V neuron, and from the outset of its development, the minor field is completely overlapped by the major field of a serially homologous neuron. Second, the axon fields of the P neurons expand in their territories only until the adult configuration is attained, early in stage 11. Thus the receptive field boundaries of the P neurons are established directly, and not by an initial overgrowth followed by elimination of the excess processes, which is a pattern observed in the developing vertebrate nervous system (e.g., Brown et al., 1976; Hubel et al., 1977; Lichtman, 1977; Rakic, 1977; Lichtman and Purves, 1980).

Detailed observations indicate that the process of receptive field development by the P_V neuron is indeed direct and highly stereotyped. Receptive field development always begins with a basic axon branching pattern: a central branch that grows out first, along the middle of the future central annulus, from which perpendicular first-order longitudinal branches emerge at three predictable places in the germinal plate (the B1, B2, and B3 branches). Perpendicular second-order annular branches emerge at the middle of adjacent annuli from the first-order longitudinal branches as they elongate. From the annular branches in turn, third-order longitudinal branches emerge at closely and rather evenly spaced

Figure 7. Formation of the boundary between the major and minor field axons of the P_V neuron. All panels show neurons filled with Lucifer Yellow photographed in fixed whole mounts. *A,* Major (*ma*) field axons of P_V neurons in two adjacent midbody segments at stage 11(0/20). The intersection of the major fields of the two neurons occurs along a line between the *arrows*. B1 and B2 longitudinal branches in this and other photographs are labeled. The cell body of the *right-hand neuron* is out of the picture. *B* to *E,* Anterior minor (*mi*) and major (*ma*) field axons of P_V neurons in the 7th segment. This minor field is atypically large because it lies in the skin of the 6th (clitellar) segment. *B,* Stage 11(0/20). The boundary between major and minor field axons is along a line between the *arrows*. *C* and *D,* Stage 11(2/20). *D* is high magnification view of the major/minor field boundary of the neuron shown in *C.* The boundary is along the *dotted line.* Major and minor B1 branches appear to touch in this photograph, but examination of the whole mount revealed tips of the two branches to be in different focal planes. The same is true of branches between the third and fourth *dots* from the *bottom* of the panel. *E,* Stage 11(3/20). The boundary is along the *dotted line.* The tapered tips of the major and minor B1 branches apparently do touch. *Calibration: A* to *C,* and *E,* 100 μm; *D,* 50 μm.
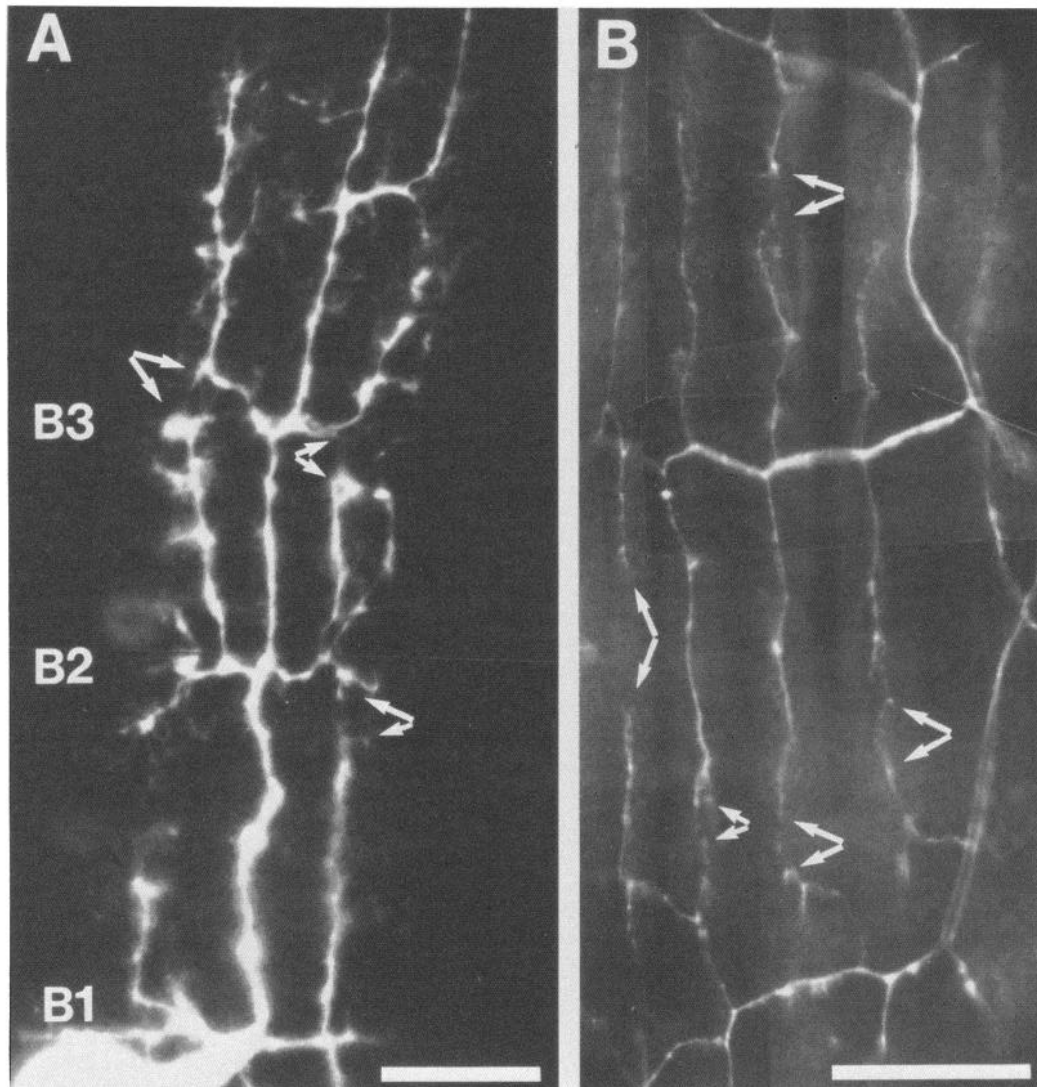
Figure 9. Growth-limiting self-encounters of P_V neuron axons, as seen among peripheral axons of Lucifer Yellow-filled P_V neurons photographed in whole mount. *A*, The major field axons at stage 10(2/5). Laterally directed second-order annular branches from B1 and B2 branches encounter the B2 and B3 branches, respectively (at *arrows*). These axons are spatially separated, but focusing through the preparation revealed apparent filopodial contacts between the encountering branches; filopodia projecting from ends of axons are blurred in the photograph. *B*, Encounter of fourth-order circular branches in a neuron in the midbody at stage 11(2/20). Circular branches growing from neighboring third-order longitudinal branches along the same circumferential path encounter each other at *arrows* (that point to ends of axons) and are separated. Blurred filopodia are visible between the ends of some pairs of circular branches. The thick, faintly fluorescent circumferential stripe beneath each row of circular branches is an autofluorescent circular muscle fiber. *Calibration*, 50 μm.

positions, and from these, finally, fourth-order circular branches grow out along specific circumferential pathways and arborize in the epidermis to form sensory endings. Except for a few short first-order longitudinal branches which may grow from the central branch before the basic branching pattern is completed, virtually all of the axon branches that are extended appear to form part of the late embryonic (essentially mature) field structure.

This stereotyped pattern of development is so robust that in segments where the B1p branch is missing and is replaced by a secondary axon that has emerged directly from the CNS (Fig. 10A), that secondary axon develops the branching pattern typical for a B1p branch. One interpretation of this stereotyped pattern of receptive field development is that the peripheral axons of the P_V

neuron grow along prespecified pathways. Muscle fibers might be important components of such pathways, since the embryonic branching pattern of the peripheral axons of the P_V neuron bears a close relation to the orthogonal grid of primordial longitudinal and circular muscle fibers in the germinal plate (Fig. 4; Kramer and Stuart, 1982). This interpretation is supported by the observation that the primary axon of the P_D neuron apparently grows along a pathway of particular muscle and other mesodermal cells (Kuwada, 1982; Kuwada and Kramer, 1983).

Determinants of subfield structure. In the development of some nervous systems, an orderly temporal sequence of outgrowth of axons from the neurons has been shown to be responsible for the formation of appropriate neuronal connections (e.g., Anderson, 1978; Macagno, 1978).
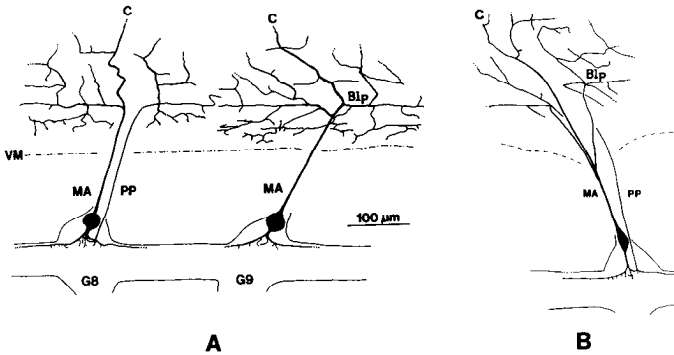
A   **B**

Figure 10. Variations in the development of secondary axon subfields of P_V neurons; tracings from photographs of neurons filled with Lucifer Yellow. *A*, Two P_V neurons in adjacent ganglia of a stage 11(0/20) embryo. The P_V neuron in the 8th abdominal ganglion (*G8*) has a secondary (*PP*) axon with a large arborization in the posterior segmental germinal plate. The primary and secondary axon arborizations are completely non-overlapping. The primary (*MA*) axon lacks a B1p branch. The P_V neuron in *G9* lacks a secondary axon, and its primary axon (*MA*) has a B1p branch that occupies a territory homologous to that occupied by the secondary axon from P_V in *G8*. *B*, P_V neuron in a different, stage 10(4/5) embryo. This neuron has a secondary axon, but that axon has not formed an appreciable arborization in the germinal plate. However, the primary axon of this neuron has a B1p branch with its normal arborization.

In the case of the primary axons of the P_V and P_D neurons, temporal differences of outgrowth between them do not appear to determine which territory of segmental skin each will innervate, since both neurons initiate growth of their primary axon at about the same time (Kuwada and Kramer, 1983) and begin to innervate their respective peripheral territories at the same time (Fig. 2*A*). However, temporal differences of outgrowth between primary and secondary axons of the same neuron might very well determine the territory where each axon will form its subfield. For instance, the primary axon of the P_D neuron, which is first to emerge, grows directly to the dorsal germinal plate, and the secondary axons, which emerge later, grow directly into the ventral germinal plate. Thus, the first axons of the P_D neuron bypass the ventral germinal plate, even though they initially contact it, whereas later axons innervate the ventral plate. Timing of outgrowth could play a role in this differential innervation pattern if, for instance, the P_D neuron switches its preference from dorsal to ventral territories between the time of primary and secondary axon outgrowth, or if the attractiveness of the ventral territory for the P_D neuron increases between the two periods of axon outgrowth.

The timing of axon outgrowth is likely to determine also the size and shape of neuronal subfields. Thus, in the case of the P_V neuron, the primary axon grows out before either the minor field axons or the secondary axon, and the primary subfield it forms is larger than either the minor subfields or the secondary subfield. Furthermore, it appears that the secondary axon sometimes grows out before the B1p branch is extended, in which case the secondary subfield replaces the subfield of the B1p branch, and when the reverse is the case, the

B1p branch subfield is larger than the secondary subfield, if it is present. Similarly, we propose that the first annular branch extended from a B2 branch sometimes precedes the B3 branch into territory normally occupied by a B3 branch, with the result that the subfield of the B3 branch either is missing or shifted to another territory. Thus subfield axons appear to occupy peripheral territory on a first-come-first-served basis. That is to say, each new branch of an axon of the P_V neuron grows only into a place in the periphery that is not occupied by another branch of the same neuron. This mutual territorial exclusion of axon branches of the same neuron (which we designate as *isoneuronal* branches) can be regarded as the consequence of a developmental rule, which can be inferred from our observations: isoneuronal branches do not grow across each other on the same substrate (Figs. 7, *B* to *E*, and 9). By contrast, branches belonging to different, though serially or contralaterally homologous neurons (which we designate as *heteroneuronal* branches) are not subject to that restriction (Fig. 7*A*). The sharp boundaries between the subfields of a mechanosensory neuron are attributable to this rule. As seen in Figure 8, the developing major field of the P_V neuron continues to expand longitudinally, even as it interpenetrates the major field of the serially homologous P_V neuron in an adjacent segment. However, expansion stops as the major field branches encounter their isoneuronal minor field branches growing in the adjacent segment.

The source of the mutual territorial exclusion by isoneuronal branches could, in principle, be a competition among isoneuronal branches for a limited number of peripheral targets. In this case, each individual neuron would have to have its own set of targets that are labeled to designate them for just that one neuron so that homologous heteroneuronal branches that share the same territory would not compete. This seems an unlikely possibility because isoneuronal mutual exclusion is already manifest at the earliest stages of axon outgrowth, when the axons are growing in the muscle layer, long before they reach their epidermal targets. Thus a more attractive explanation is that isoneuronal, but not homologous heteroneuronal, axons avoid each other when growing on the same substrate (Kramer, 1982). This self-avoidance would require that processes projected by a neuron be able to distinguish between self and non-self. This self-recognition of two growing axonal processes might be mediated by their numerous filopodia, which may reach lengths up to 50 μm and do appear to make mutual contacts. In line with this possibility, it has been proposed that filopodia play an important role in the recognition and choice of axonal growth pathways by insect neurons (Goodman et al., 1982; Ho and Goodman, 1982; Taghert et al., 1982; Keshishian and Bentley, 1983; Raper et al., 1983a, b).

Non-overlap of isoneuronal processes could be a general phenomenon of neuronal development, since the adult morphology of many neurons appears to satisfy the rule that isoneuronal processes do not cross each other on the same substrate. This property is especially apparent in the leech mechanosensory neurons, because each neuron has several distinct axons that innervate neigh-

boring regions in the same spatial plane. Other neurons with two-dimensional fields of processes for which this property is similarly evident are alpha cells of retina and cerebellar Purkinje cells. The functional role for such a "self-avoidance" phenomenon could be, for instance, to ensure an optimal coverage of the available target territory.

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