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Developmental Arborization of Sensory Neurons in the Leech *Haementeria ghilianii*

II. Experimentally Induced Variations in the Branching Pattern[1]

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Abstract

The sharp, nonoverlapping boundaries of the major and minor receptive fields of the mechanosensory neuron P_V of the leech, as well as the mutual exclusion during embryonic development of growing axon branches belonging to the same P_V cell, have suggested that peripheral axon arborization of these neurons is constrained by a process of neuronal self-avoidance. To provide a direct experimental test of this proposal, the development of the major and minor receptive fields of the P_V neuron was studied in embryos of the leech, *Haementeria ghilianii*, after surgically preventing or delaying the outgrowth of the axon branches which establish only a minor or only the major field of that neuron. As predicted by the proposal of self-avoidance, interference with the outgrowth of a minor field axon branch resulted in the spread of the major field axon branch into what is normally minor field territory. Conversely, similar interference with the establishment of the major field resulted in the spread of the minor field axon branches into what is normally major field territory. The findings presented here indicate that neuronal self-avoidance does play a significant role in the development of mechanosensory receptive field structure but suggest also that the detailed pattern of arborization of the sensory axons is guided by prespecified pathways of only ephemeral availability or recognizability.

Previous studies of the development of the peripheral arborization of leech mechanosensory axons have shown that axon branches belonging to the same sensory cell, designated *isoneuronal* branches, almost never contact each other, and that they never grow past one another on the same guidance pathway. These axon branches do, however, freely overlap with the growing axon branches of serially and contralaterally homologous sensory cells, designated as *heteroneuronal* branches, that occupy some of the same peripheral territory. These observations have led to the proposal that, during embryonic development, the growing axons of these mechanosensory neurons are subject to a process of self-avoidance (Kramer, 1982; Kramer and Kuwada, 1983). Self-avoidance would account for the sharp, nonoverlapping boundaries that separate various domains of the mechanosensory fields innervated by different isoneuronal axon branches (Kramer and Goldman, 1981; Kramer et al., 1985) and would require that an axon be able to distinguish its isoneuronal from heteroneuronal branches that it encounters while aborizing in the skin.

The experiments reported here provide a direct experimental test of the importance of neuronal self-avoidance in the establishment of nonoverlapping mechanosensory fields in embryos of the glossiphoniid leech, *Haementeria ghilianii*. Each of the segmental ganglia of the leech contains a bilateral pair of identified mechanosensory, or "pressure," neurons designated as the P_V neurons (Nicholls and Baylor, 1968). The receptive field of the P_V neuron lies in the ventral skin, on the same side as its cell body, and consists of one major field and two minor fields (Yau, 1976). The major and minor sensory fields are innervated by axon branches of the P_V cell, which exit from three different segmental ganglia, and are themselves composed of distinct subfields, innervated by axon branches which course in different peripheral nerves. Major and minor fields, as well as their subfields, are separated from each other by sharp boundaries, across which there is no overlap (Nicholls and Baylor, 1968; Yau, 1976; Kramer and Goldman, 1981; Kramer et al., 1985). The axonal arborization of a P_V neuron can be visualized at various stages of leech development by dissecting an embryo, penetrating the cell body neuron with a microelectrode, and filling it with the fluorescent dye Lucifer Yellow (Kramer and Kuwada, 1983; Kuwada and Kramer, 1983). Such studies have shown that the P_V mechanosensory neuron extends its first axon (the major field axon branch) from the ganglion containing the cell body into the periphery on the third day of embryonic stage 9. The minor field axon branches exit from adjacent ganglia on the first day of stage 10, i.e., about 4 days later.

This paper reports experiments in which either major or minor field axons were delayed in—or in some cases prevented from—their embryonic outgrowth by surgically crushing particular nerve roots at the beginning of stage 10. The morphology of the P_V neuron was then examined during early stage 11, at which time the cell normally has elaborated a branching pattern comparable to that seen in the adult leech. If the boundaries between subfields were established and maintained by a process of axonal self-avoidance, it would be predicted that the experimental prevention or delay of outgrowth of a given P_V axon branch should result in the spread of that neuron's other axon branches into the territory normally occupied by the absent or delayed branch. The results presented here meet this prediction.

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Materials and Methods

The source of embryonic and adult specimens of *Haementeria ghilianii* and the staging of embryos according to a set of external morphological characteristics were the same as those previously described (Fernandez, 1980; Weisblat et al., 1980; Stent et al., 1982; Kramer et al., 1985).

Preparation of operated embryos. Stage 10(0/5) to 10(2/5) embryos were anesthetized in a cold solution of 0.15% chlorobutanol in *Haementeria* colony water (Sawyer et al., 1983) for 10 min and were then transferred to a Sylgard bottom dish containing cold leech physiological saline solution (Kramer and Goodman, 1981). To improve visualization of the nerve cord and peripheral nerves, a saline solution containing 1% Fast Green dye was injected into the coelomic cavity of the embryonic germinal plate with a fine glass needle. The preparation was immobilized, ventral side up, by pressing it against the Sylgard surface with staple-like pins across its head and tail. Forceps were used to tear a small opening in the body wall overlying a hemiganglion and its peripheral nerve roots. The forceps were used to pinch both anterior and posterior nerves or the anterior nerve alone, depending on the experiment. Pinching resulted either in a crush or a complete cut of the nerve. Crushes usually allowed delayed outgrowth of axons, whereas cuts often prevented any further outgrowth of the P_V axon branch along that nerve. Generally, four or five, but sometimes as many as eight midbody ganglia (G6 to G15), were operated in each embryo. After the operation, embryos were allowed to rewarm in a saline solution for 15 to 30 min. They were then transferred to *Haementeria* colony water containing 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 20 μg/ml of tetracycline hydrochloride, and allowed to continue development at 27°C, with daily solution changes. The operation resulted in some loss of coelomic fluid and, occasionally, loss of yolk, due to accidental punctures of the gut membrane, but the embryos healed quickly and continued to develop without gross abnormalities. Development was also unaffected by the Fast Green dye, which was largely purged from the embryo within a few days after injection.

The operated embryos were dissected after they had reached stages 11(0/20) to 11(5/20). They were prepared and examined as previously described (Kuwada and Kramer, 1983), with the following exceptions. Before dissection, embryos were relaxed for 10 min in 0.15% Chlorobutanol saline solution. After dissection, they were examined in physiological saline solution containing 15 mM Ca^{2+} and 15 mM Mg^{2+} (which reduces movements of the preparation). Operated hemiganglia in dissected preparations were located by means of a compound microscope with Nomarski optics. Visual inspection revealed whether peripheral nerves were missing or damaged, as indicated by their abnormally small width (e.g., Fig. 7). Occasionally, anterior and posterior nerves in operated ganglia had fused, but unless they were also of small width, the P_V neuron peripheral axons usually had not been damaged. After visual confirmation of peripheral nerve damage, the P_V neuron located in the operated ganglion, or in an immediately adjacent anterior or posterior ganglion, was injected intracellularly with a 5% solution of Lucifer Yellow dye (Aldrich). Preparations were fixed for 16 to 24 hr at 4°C in 4% paraformaldehyde, buffered to pH 7.4 with HEPES. The fixed preparations were rinsed in saline, whole mounted under a coverslip in saline, and immediately examined and photographed. The peripheral arborization of the neurons was examined under epi-illumination in fixed whole mounts. As a control, 10 hemiganglia were sham-operated; the germinal plate was torn open to expose the hemiganglion nerves, but they were not pinched. The P_V neurons with either a major or minor field axon branch in the segmental nerves of the sham-operated hemiganglion all developed normal arborizations (as shown by comparison of Fig. 1 with Fig. 7 of Kramer and Kuwada, 1983).

Results

Normal pattern of sensory field innervation. During their normal outgrowth, the P_V axons follow particular pathways and branch at specific sites in the body wall. As they first leave the ganglion, the axons follow the circumferential rings, or annuli, into which the skin of each body segment is divided. In *H. ghilianii*, the dorsal and ventral skin is divided into three and five annuli, respectively, per midbody segment. The segments are defined with reference to the ganglia, with the annulus aligned with the ganglion being designated the central annulus. The major field axon branch of cell P_V grows out of the ganglion circumferentially (i.e., perpendicular to the long axis of the body) along a straight pathway in the region of the central annulus. Similarly, the minor field axon branches later grow out circumferentially along the central annulus region of the next anterior and posterior adjacent segments. These first outgrowths are termed the *central branches* of the major and minor field axons. In about one-third of *H. ghilianii* embryos, there are two major field axon branches, which grow out in different nerves. In these cases, it is always the branch which grows out first, or the *primary* axon branch that establishes the central branch.

Having reached the periphery, the major or minor field axon branches undergo further branchings, with many of the higher-order branches also growing along specific pathways. Among these are three first-order longitudinal branches, designated B1, B2, and B3, which are extended perpendicularly from the central branch at each of three circumferential positions in the body wall. They, in turn, extend second-order circular branches which grow circumferentially down the middle of the other annuli. Then, third-order longitudinal branches are extended, followed by fourth-order circular branches. These features of the normal axonal arborization pattern can be seen in the ganglion body wall preparation from an early stage 11 embryo in which a P_V cell has been filled with Lucifer Yellow dye (Fig. 1).

Another feature which is manifest in the preparation shown in Figure 1 is that, as previously described (Kramer and Kuwada, 1983), none of the branches of a P_V neuron arborization overlap on common growth pathways. In particular, the branches of adjacent major and minor field arborizations do not overlap, so that there is a sharp boundary between them. This boundary always falls somewhere between the two central annuli of adjacent segments, so that the major field branches normally never enter the central annulus territory of the adjacent segment (Kramer et al., 1985).

Spread of major field axon branches into minor field territory. To determine whether the minor field arborization excludes the major field axon branches from extending into its territory, the nerve containing a minor field axon branch of the P_V neuron was crushed just as this neuron began to extend into the periphery in a series of early stage 10 embryos. These embryos were then raised to early stage 11, by which time major and minor fields would normally display a common boundary, and the P_V cell was filled with dye to examine its branching pattern.

The results of one such experiment are shown in Figure 2. The anterior minor field arborization is abnormally small, suggesting that the outgrowth of this neuron was retarded by the crush. The major field axon branch has spread into the normal minor field territory. The first-order longitudinal B1 axon branch has extended into the central annulus of the operated segment and there has given rise to a large, second-order circular branch. The results of another such experiment are shown in Figure 3. Here the anterior minor field axon does not extend into the periphery, and it can be seen that the B1 branch of the major field has spread only into the anterior segment, which is missing the minor field branches. By contrast, in the posterior (unoperated) segment, where minor field branches are present, the major and minor field arborizations occupy their normal territories and display a sharp boundary.

The specimen presented in Figure 4 exemplifies another pattern of major field axon spreading produced by delay of outgrowth of minor field axons. In this embryo neither of the two P_V cells in the anterior and posterior ganglia flanking the ganglion whose peripheral nerves had been cut extended the B1 branch of their major fields into the central annulus region of the vacated territory. However, the P_V cell in the anterior ganglion had extended its longitudinal B2 branch into the more distal sector of the vacated central annulus region, where it gave rise to a large, second-order circular branch. For its part, the P_V cell in the posterior ganglion had invaded only even more distal sectors of the central annulus region, by means of third-order longitudinal branches.

Heteroneuronal influence on axon branch spreading. Since the minor field axon branch of one P_V cell and the major field axon branch of its serial homologue in the adjacent segment course in the same peripheral nerve, crushing this nerve should perturb both axons. Thus, these major field axons are spreading into the territory normally occupied by both their own minor fields and the major field of homologous P_V neurons in the adjacent segment. To determine

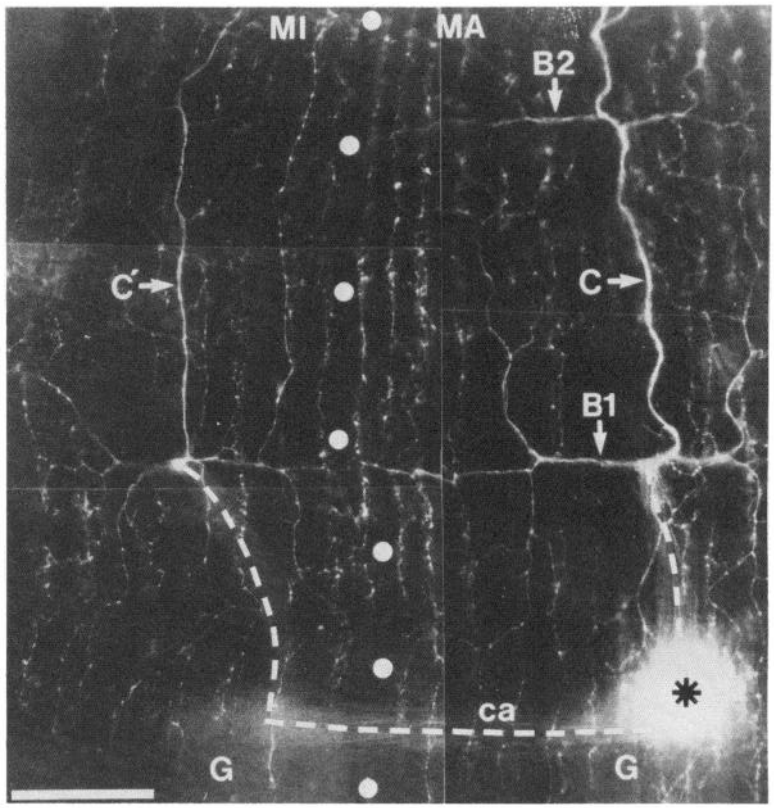

Figure 1. Arborizations of major and minor field axon branches of a normal P_V neuron whose minor field hemiganglion was sham-operated. Shown is a fluorescence photomicrograph of a midbody neuron filled with Lucifer Yellow in a whole mount preparation at stage 11(3/20). In this and all other figures, anterior is to the *left* and fluorescent axon branches which are out of focus are indicated by *dashed lines*. The cell body is under the *asterisk* (out of focus region) in the ganglion (*G*). The neuron extends a peripheral axon branch from its own ganglion that arborizes in the ipsilateral ventral body wall of its segment to form the major subfield (*MA*, to the *right* of the *dotted line*). The neuron also projects an axon in the connective nerve (*ca*) to the next anterior ganglion, where it extends a peripheral axon branch to form the anterior minor field in the adjacent segment (*MI*, to the *left* of the *dotted line*). The central branches of the major and minor field axon branches grow in the middle of each segment along the central annuli (*C* and *C′*) of the segmental body wall. First-order longitudinal branches (*B1* and *B2*) extend from the central branch. Also present are second-order annular branches, third-order longitudinal branches, and fourth-order circular branches, which form in close parallel circumferential rows. The *dotted line* is the nonoverlapping boundary between major and minor fields. None of the branches growing along the same pathway overlap; fourth-order branches only seem to overlap longitudinal branches because they are growing in different planes of the body wall. *Calibration bar*, 100 μm.

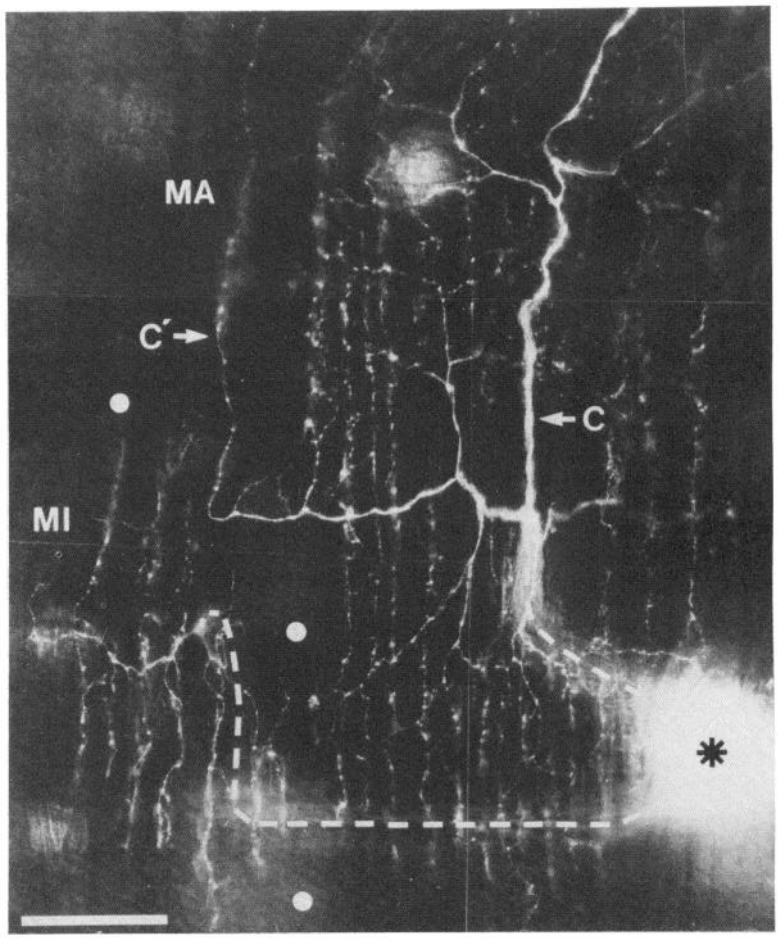

Figure 2. Spread of first-order axonal branches of the major field branch of an operated P_V neuron into experimentally vacated minor field territory. This P_V neuron belongs to the same specimen as that shown in Figure 1, and the same labeling conventions are used. The anterior minor field axon was crushed 7.5 days earlier at stage 10(0.5/5), delaying its outgrowth. The major field axon and its arborization have spread into the central annulus region (*C′*) that is normally occupied by the minor field axon. The central branch pathway of the anterior segment (*C′*) is occupied by a branch of the first-order B1 branch of the major field branch. *Calibration bar*, 100 μm.

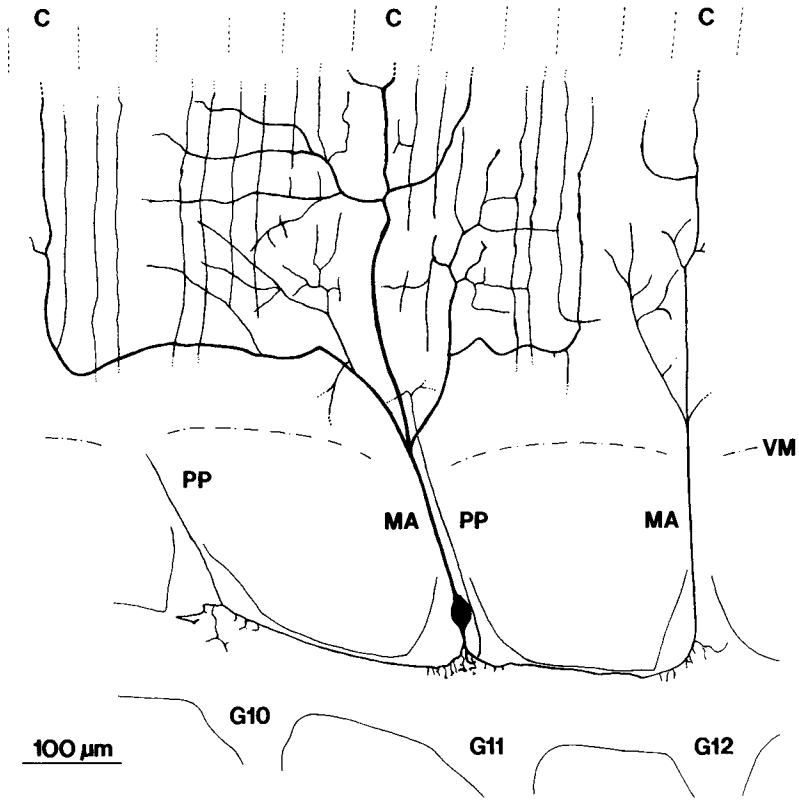

Figure 3. Spread of major field arborization into the operated (anterior) minor field P_V but not into the unoperated (posterior) minor field. The drawing is from photographs of a stage 11(4/20) P_V neuron in ganglion 11 (*G11*) of an embryo whose minor field axon, extended from ganglion 10 (*G10*), had been crushed 7 days earlier at stage 10(2/5). In this preparation, the body wall was cut along the ventral midline (*VM*) and pulled away laterally from its normal location under the nerve cord. Normally, the primary subfield axon branch is located in the MA nerve and the secondary subfield axon branch is located in the PP nerve. The anterior minor field axon branch which had been crushed was extended via the PP rather than the normal MA nerve and had not yet arborized in the body wall. *Fine dashed lines* are boundaries of the skin annuli with *C* designating the central annulus of each segment.

whether spreading is influenced by damage of the serial homologue's major field, nerve crush operations were carried out on a series of 17 embryonic ganglia, and the axonal arborizations of both the P_V cell in the operated ganglion and its serial homologue in the adjacent ganglion were ascertained at a later stage. These experiments show that the different patterns of major field spreading described in the preceding section are correlated with the degree to which the operation also eliminated the major field arborization of that serially homologous P_V neuron in the adjacent segment.

The result of one such experiment is presented in Figure 5. This case resembles the anterior P_V cell in the preparation of Figure 4 in that, here, too, the cell whose minor field axon branch had been crushed extended only its distal B2, but not its proximal B1, first-order longitudinal branch into the vacated central annulus region of the adjacent segment. Dye fills of the serially homologous P_V neuron showed that, following a nerve crush, its major field axon had extended a normal first-order longitudinal branch only at the B2 position, but not at the B1 position. That is to say, the major field axon of one P_V neuron invaded only those vacated portions of its minor field in which the major field axons of the serially homologous axons were also present. Moreover, far from avoiding each other, the serially homologous axon branches grew along each other.

In 5 of the 17 cases examined, the P_V neuron whose minor field outgrowth was retarded by nerve crush extended B1 or B2 first-order longitudinal branches from the major field into the central annulus region of the adjacent segment with the crushed peripheral nerve. In 4 of these 5 cases, the serially homologous P_V cell had developed a large major field arborization following the crush of its own major field axon, and this arborization overlapped with the major field axons which had spread into the territory normally occupied by the minor field. In 1 of these 5 cases, the serially homologous P_V had failed to develop a large major field arborization following the crush of its major field axon branch. The remaining 12 cases resembled the posterior P_V cell in the preparation of Figure 4, in that there was only little spread of major field axon branches into the vacated minor field territory, and what spread did occur resulted from outgrowth of third-order, rather than first-order, axon branches. In all of these 12 cases following the crush of its axon, arborization of the major field of the serially homologous P_V cell was either completely missing or very small and did not overlap with the major field axon branches of the other P_V cell. Thus, the spread of major field axon branches B1 and B2 after elimination of the minor field is associated, not with the absence of the adjacent homologous major field, but with its presence.

Spread of minor axon branches into major field territory. A further prediction of the theory of neuronal self-avoidance is that minor field axon branches should spread into regions normally occupied by the major field axon branches when outgrowth of these major field branches is delayed. To test this prediction, major field axon branches already extended from P_V cells of early stage 10 embryos were crushed and the axonal arborization pattern of these cells was examined at a later stage. The result of such an experiment is shown in Figure 6. As predicted, following the crush of the major field axon branch and its delayed regrowth, the major field was smaller and the minor field larger than is normally the case, with the boundary between major and minor fields being located far within the territory normally occupied by the major field. Figure 8 presents a similar result in a case, to be discussed below, in which the primary major subfield axon had been cut, leaving only a smaller secondary major subfield.

Spread of secondary axons into primary subfield territory. In about 30% of P_V neurons the major field is innervated not only from the regular primary major field axon branch which exits the ganglion via the major (MA) nerve but also by a variable secondary axon branch that exits the ganglion via the PP nerve (Kramer et al., 1985). The subfield of the major field maintained by the secondary axon branch is located in the posterior part of the segmental skin. The subfield maintained by the secondary axon branch is usually very small compared to the subfield maintained by the primary axon branch, and it does not grow along the territory of the central annulus, as illustrated in the specimens shown in Figures 3 and 7. The theory of neuronal self-avoidance predicts that the secondary PP subfield axon branch of the P_V neuron should spread into the primary subfield territory if that territory is vacated by cutting the

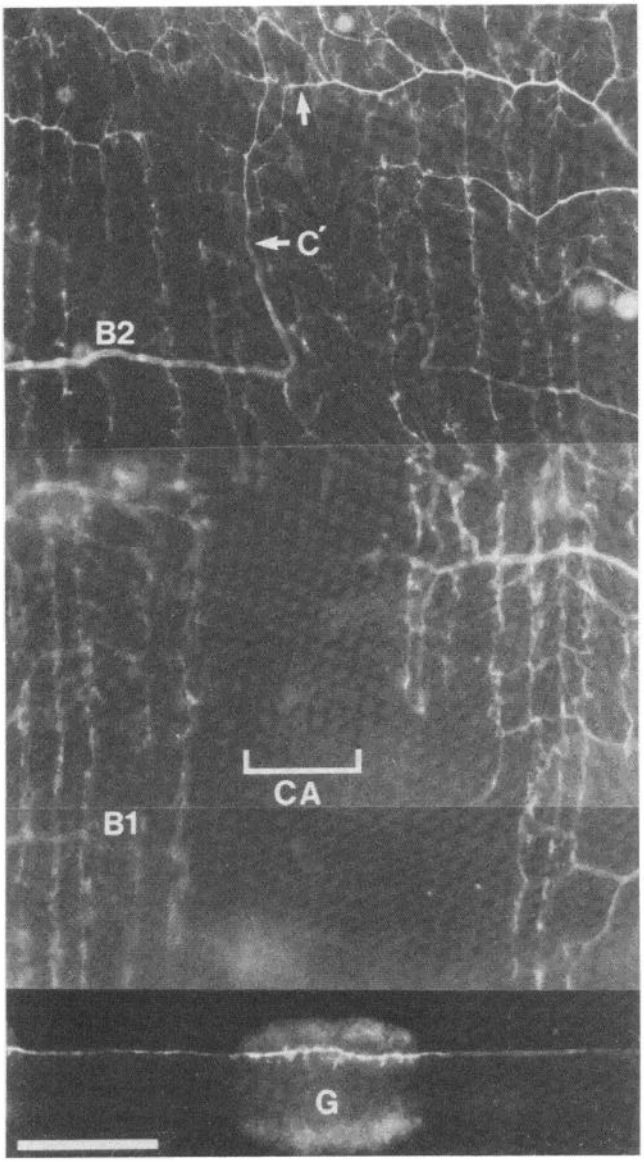

Figure 4. A different pattern of spread of the major field into the vacated adjacent central annulus region. The peripheral nerves in one ganglion (*G*) had been cut 5 days earlier at stage 10(2/5). The P_v neurons (ipsilateral to the cut) in the anterior and posterior adjacent ganglia were filled with Lucifer Yellow dye. Neither P_v neuron has extended a minor field axon from the operated ganglion by stage 11(2/20), nor has the major field axon branch of either neuron invaded the vacant central annulus region (*CA*) proximal to the ganglion at the level of the B1 branches. However, the B2 branch of the major field of the anterior P_v neuron has spread into the vacated central annulus region and extends a circumferential branch (*C'*) along a distal part of the central annulus. The major field of the posterior P_v neuron enters the vacated central annulus region only via distal third-order longitudinal branches (at *unlabeled arrow*). *Calibration bar*, 100 μm.

primary MA axon branch. This prediction can be tested experimentally, because it is possible to crush only the MA nerve containing the primary subfield axon branch, while leaving intact the PP nerve containing the secondary subfield axon branch (Fig. 7). The axonal arborization pattern resulting from this operation, presented in Figure 8, confirms the prediction of the self-avoidance theory. The secondary PP subfield arborization has increased dramatically, occupies the central annulus region (C), and has spread to the anterior part of the segment. Moreover, the operation has also increased the frequency of P_v cells in which the secondary PP axon participates at all in the formation of the major field from the normal frequency of about 30% to about 90% (i.e., 11 of 12 cases).

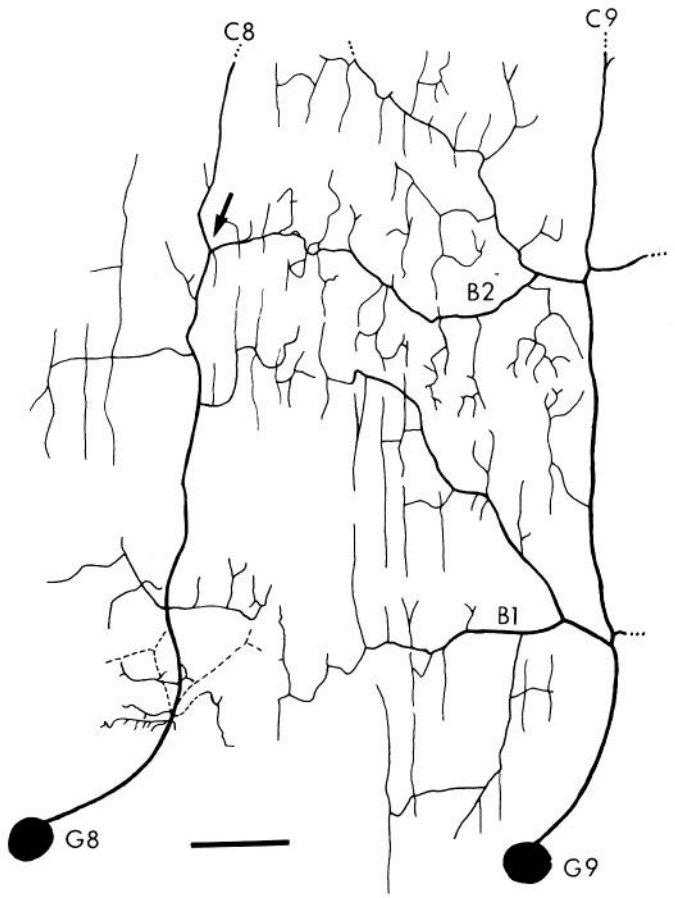

Figure 5. Spread of major field first-order axon branches of an operated P_v neuron in association with major field first-order axon branches of a serially homologous P_v neuron. The peripheral nerves of the eighth ganglion (*G8*) were crushed at stage 10(1/5). At stage 11(4/20), the embryo was dissected, and the P_v cell of *G9* (that is, P_v (9)) was filled with Lucifer Yellow dye and its axonal branching pattern was recorded. The anterior minor field of $P_v(9)$ in segment 8 is missing; the first-order longitudinal branch B1 of $P_v(9)$ has grown into segment 8, but it did not invade its central annulus region (C8), having previously left the normal B1 pathway and terminated as a circular branch. By contrast, the more distal first-order longitudinal branch B2 has spread into the C8 region, extending to the position marked by the *solid arrow*. After recording this branching pattern, the dye was bleached by prolonged illumination. The homologous cell $P_v(8)$ (*G8*) was then filled with dye, and its processes fluoresced much more brightly than, and could be readily distinguished from, those of $P_v(9)$. The major field axon of $P_v(8)$ grew along C8 but did not extend a B1 branch. Its arborization pattern in the B1 region proximal to the ganglion is very disorganized and includes abnormal branches apparently lying on the interior surface of the longitudinal muscles (*dashed lines*). No branches of $P_v(8)$ overlap with the B1 field of $P_v(9)$ or with its higher-order branches. More distally, $P_v(8)$ has extended a posteriorly directed first-order longitudinal branch at the normal B2 position, which extends along the B2 branch of $P_v(9)$. Along almost the whole course of their co-extension, the B2 axon branches of the two homologous neurons follow the same route and are indistinguishable in the whole mount preparation under fluorescence microscopy at × 640 magnification.

Abnormal branching patterns of delayed subfield axons. It is a striking feature of the preparations examined here that axon branches whose outgrowth had been delayed by a nerve crush failed to grow along many of the pathways normally followed. The delayed branches often followed an unusual route, leaving some normal first- and second-order branch pathways vacant. For example, neither the delayed minor field axon of Figure 2 nor the delayed major field axon of Figure 6 formed first-order longitudinal branches along the normal B1 and B2 pathways. However, in all cases, the delayed axon branches did occupy the pathways normally occupied by the fourth-order circular branches, although, in the delayed axon

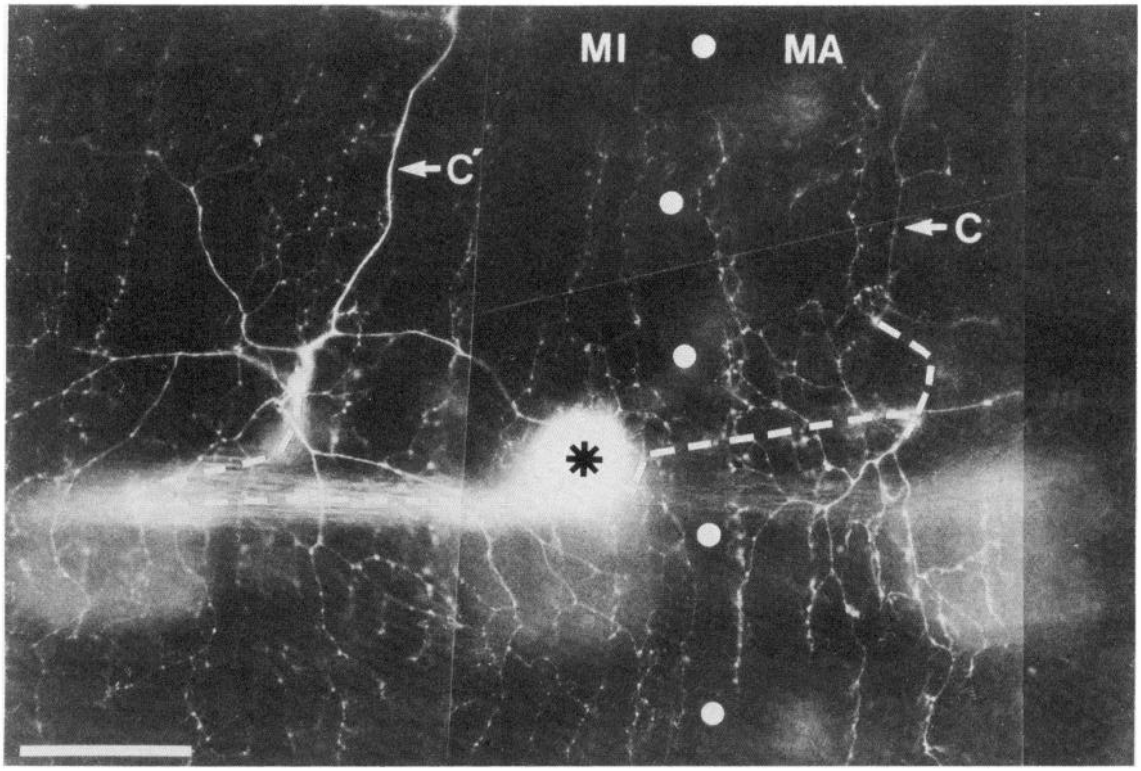

Figure 6. Surgically induced spread of minor field axons into major field territory. This P_V neuron (in G7) belongs to the same specimen as the neurons shown in Figures 1 and 2, and the same labeling conventions are used. The major field axon (*MA*) was crushed 7.5 days earlier at stage 10(0.5/5), delaying its outgrowth. The minor field (*MI*) lies in the skin of the sixth, or clitellar, segment. Although minor fields in the skin of the clitellar segment are normally much larger than in the skin of other segments, they do not ordinarily extend into the segmental skin of the major subfield. However, in this operated specimen, the minor field extends into the skin of the major field segment by the width of at least one annulus. The major field branch, *C*, lies in the central annulus of the segmental body wall, but it is a fourth-order circular branch rather than the main branch which is normally located in the central annulus.

branches that occupied these pathways, these branches were not always of the fourth order (e.g., Fig. 2).

Discussion

The mechanism of neuronal self-avoidance. The findings reported here indicate that development of peripheral axon arborization of the mechanosensory P_V neuron is governed by a process of territorial self-avoidance. The theory of self-avoidance by isoneuronal processes had been invoked initially to account for the sharp, nonoverlapping boundary between major and minor fields in the overall receptive field of the P_V neuron. The present experiments now demonstrate that, in accord with the self-avoidance theory, the location of this boundary can be shifted experimentally by delaying or preventing the developmental outgrowth of either the minor or the major field axon branches. In this way, either field can be made to usurp territory that normally is occupied exclusively by the other.

The main reason for invoking the notion of neuronal self-avoidance in the first place is that in embryonic mechanosensory neuron development a mutual territorial exclusion obtains only for isoneuronal but not for heteroneuronal, albeit closely homologous, axon branches. The experiments of the type presented in Figure 5 support this idea, in that they show that isoneuronal exclusion of major and minor field axon branches in embryonic development of the mechanosensory neuron is coupled with a heteroneuronal *facilitation*, between the axon branches of serially homologous P_V neurons. Hence, it is likely that the heteroneuronal exclusionary interactions recently found to exist in *adult* leeches between axon branches of homologous mechanosensory neurons maintaining receptive fields in adjacent skin territories (Blackshaw et al., 1982) reflect the operation of a different avoidance or competition process.

We may now consider the mechanism which might provide a mechanosensory neuron with the self-identity that would permit one of its growing axonal processes to recognize another process as being iso- or heteroneuronal. One possibility would be that the peripheral axons carry cell-specific, or idiosyncratic, labels. The number of such labels would have to be quite large. Each P_D neuron shares its receptive field with five other P_D neurons and five other P_V neurons, not to speak of the many T and N mechanosensory neurons with which its field also overlaps. Each of these cells would have to carry a different set of labels that would allow its own isoneuronal recognition to occur independently of any heteroneuronal interactions.

The required complexity of this idiosyncratic labeling system makes another alternative more attractive: growing processes of mechanosensory cells may identify each other as being either iso- or heteroneuronal by comparing their pattern of spontaneous impulse activity. In the case of adult mechanosensory cells, an impulse evoked at any point of its extensive axonal and dendritic arborization will generally travel through the entire system within about 50 msec. Since the P_V cell has become electrically excitable by the time of outgrowth of its processes in mid-stage 10 of development (Kramer and Kuwada, 1983), each embryonic P_V neuron could have an idiosyncratic, spontaneous activity pattern, which is temporally coherent throughout its arborization at the time that its axonal branches are undergoing self-avoidance. The random spontaneous impulse activity occurring in heteroneuronal embryonic processes would, by contrast, be incoherent. The coincidence of action potentials could therefore serve as a mechanism for two approaching axon branches to recognize their iso- or heteroneuronal character. Branches with coincident action potentials (within a window of about 50 msec) would avoid each other, whereas otherwise similar processes whose activity is not consistently coincident would not show such avoidance.

The function of neuronal self-avoidance. One consequence of

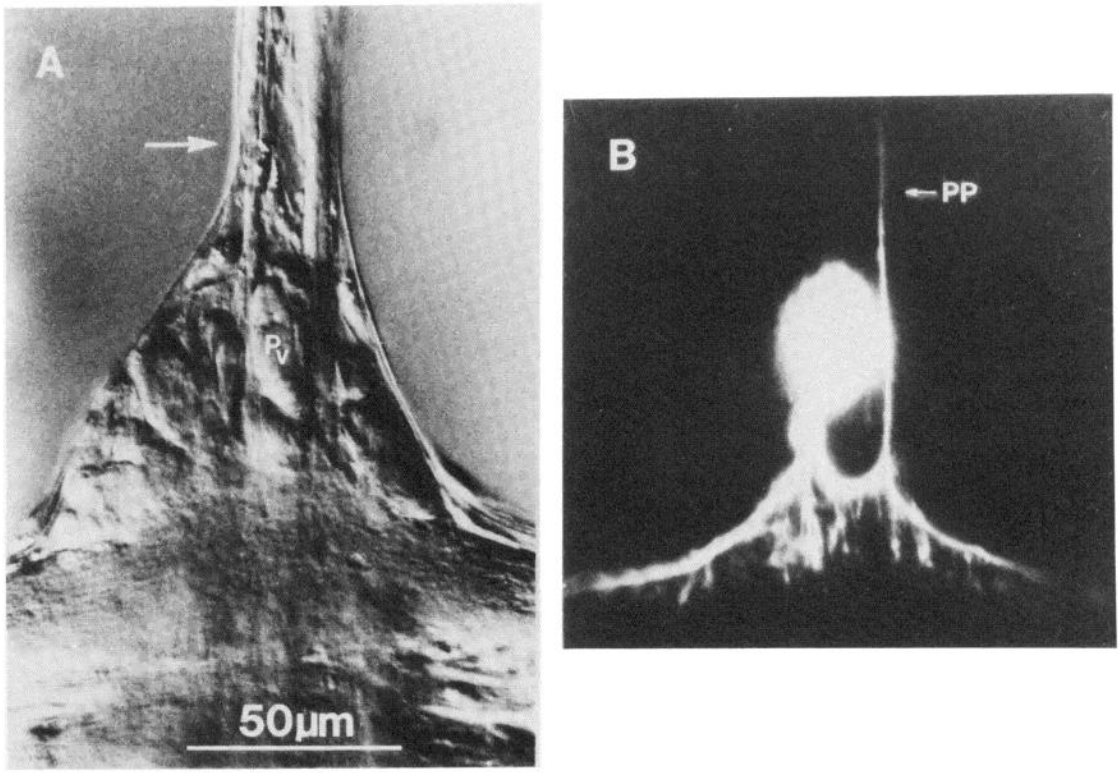

Figure 7. Crushed nerve and severed primary field axon of a P_V neuron. *A*, Nomarski optics photomicrograph of a stage 11(2/20) hemiganglion whose MA peripheral nerve had been crushed (at *arrow*) 5 days earlier at stage 10(2/5). MA is about half its normal width. The P_V neuron cell body is labeled. *B*, The P_V neuron labeled in *A*, filled with Lucifer Yellow dye. Only a secondary axon branch in the PP peripheral nerve is extended from that neuron. The large primary axon branch that would normally be extended from the cell body via the MA nerve is missing. The secondary subfield arborization of this neuron is shown in Figure 8.

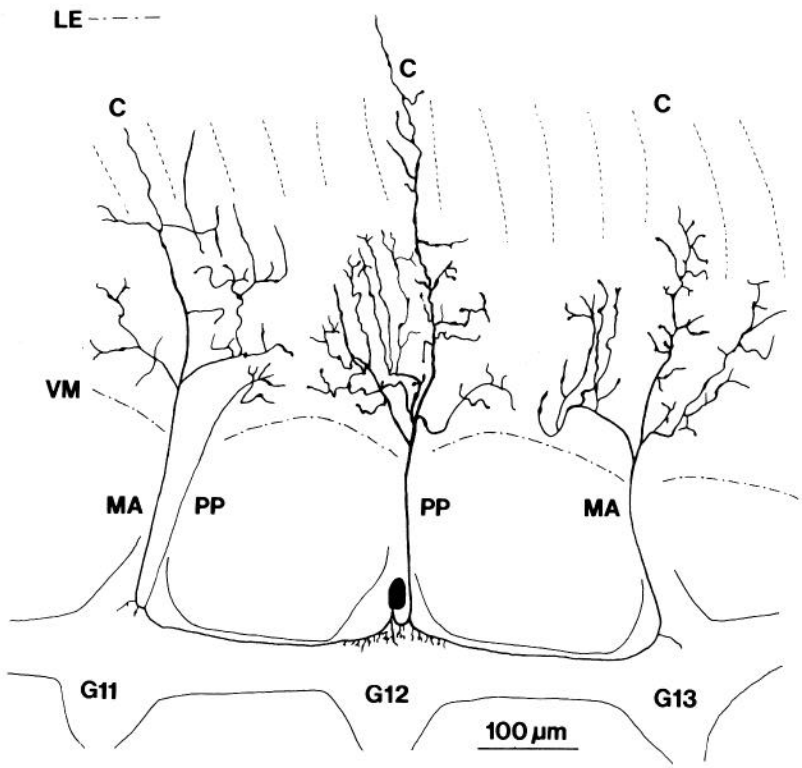

Figure 8. Spread of secondary subfield and adjacent minor field axon branches into vacated territory of the primary subfield of the major field. The drawing is of the axonal arborization of the P_V neuron shown in Figure 7, located in the 12th ganglion (*G12*). The secondary (*PP*) subfield of the major field (in segment 12), whose primary subfield axon branch (*MA*) was prevented from extending, is much larger than normal, as can be seen by comparison with Figure 3 and with the PP subfield of the minor field in segment 11 of this preparation. The minor field arborizations in segments 11 and 13 have spread into the major field of segment 12 by at least one full annulus farther than is normal.

self-avoidance, whatever its underlying mechanism, is to effect an optimal spread of outgrowing, branching processes of a neuron over a target territory which that neuron shares with the processes of one of more homologous neurons. In the case of unshared target territories, the problem of optimal spread by branching could be solved simply by the release from the growing processes of a factor which is generally inhibitory to growth of isoneuronal as well as heteroneuronal processes. Such a factor would cause branching processes to avoid, and direct their growth away from, each other and thus provide broad coverage of the target. However, this simple mechanism cannot be used in the case of targets shared by similar processes extended by homologous neurons, where a more elaborate mechanism of distinguishing self from non-self must be part of the avoidance reaction. Indeed, as will be discussed further below, since the axonal branches of homologous mechanosensory neurons appear to have an attraction for, and a tendency to grow along, each other, isoneuronal avoidance would be a necessary developmental rule to forestall self-fasciculation. This is essential, of course, because a mechanosensory neuron could not arborize if its axonal branches were attracted to each other. It seems likely, therefore, that the principal developmental role of neuronal self-avoidance is to assure the formation of optimal axonal arborization, whereas the formation of sharp, nonoverlapping boundaries between major and minor fields, whose existence first drew attention to self-avoidance, is merely one of its epiphenomena.

Prespecified developmental growth pathways. The peripheral arborization of the P_V neuron develops in a highly stereotyped spatiotemporal pattern, with the axonal branches apparently growing along prespecified pathways into the peripheral target territory (Kramer and Kuwada, 1983; A. P. Kramer and D. K. Stuart, manuscript in preparation). These growth pathways are a constant feature of the development of a given type of neuron, although there is some natural variation regarding exactly which branches of the arborization come to occupy a particular pathway (Kramer et al., 1985; Kramer and Kuwada, 1983). One such prespecified growth pathway is located along the middle of the central annulus region of each segment. Normally this pathway is followed by the primary MA subfield axon branch. But if surgical intervention prevents this pathway from being occupied by the MA subfield axon, the direction of growth of neighboring isoneuronal branches is deflected onto it (Figs. 2, 4, and 8). Thus, the stereotyped normal outgrowth pattern of the primary MA subfield axon would be the result of its being the first branch to reach the central annulus pathway. Once it occupied this pathway, it would exclude all other isoneuronal branches (Kuwada and Kramer, 1983).

One possible explanation of the abnormal arborization pattern of branching observed in the present work following experimentally induced, delayed axonal growth into the periphery (as exemplified by the failure of the MA axon to form normal B1 and B2 longitudinal branches) is that some of the prespecified growth pathways are of only transient availability. That is to say, if the growing MA axon happens to reach the B1 and B2 branch pathways at an abnormally late developmental stage, those pathways may no longer be able to perform their guiding function. Instead, growth of the delayed MA axon now follows the pathways normally followed by the third-order longitudinal and fourth-order circular branches (Kramer and Kuwada, 1983). The pathways provided by these higher-order branches, which are normally occupied only at a much later stage than those of the first-order longitudinal branches, would still be available to the experimentally delayed MA axon once it has resumed its outgrowth. Another possible explanation of these same findings would be that the pathfinding capabilities of the mechanosensory axon change with time, so that upon delayed outgrowth it can no longer follow its normal growth pathways.

The ephemeral availability of the B1 and B2 pathways or, alternatively, the ephemeral capacity of the P_V axon to follow them, would account also for the assistance rendered by the major field arborization of the homologous P_V neuron of the adjacent segment in the spread of the major field axons into its vacated isoneuronal minor field territory, as illustrated by the preparation shown in Figure 5. By the time the growing first-order longitudinal B1 branch of the spreading major field (which, as seen in Figs. 2 and 3, is the usual route by which the vacated minor field is invaded) has reached the skin of the adjacent segment, the B1 and B2 pathways may have lost their capacity for guidance. Nevertheless, if the major field of the adjacent segment has previously developed its own B1 branch, the invading B1 branch can fasciculate with its heteroneuronal B1 homologue and use it for growth guidance. (This is what would have happened in the preparations of Figs. 2 and 3. In the anterior P_V cell of the preparation of Fig. 4, an invading B2 branch would have fasciculated with its homologous B2 branch of the adjacent segment.) But if, due to its experimentally induced growth delay, the major field axon branch of the adjacent segment had failed to develop its own B1 and B2 branches, then the invading major field axon branches could have spread into the vacated minor field only via the pathways of higher-order branches. (This is what would have happened in the posterior P_V cell of the preparation of Fig. 4 and in P_V (9) of Fig. 5.)

The findings presented here thus show that, in the development of the receptive field of the P_V mechanosensory neuron, self-avoidance by isoneuronal processes plays a significant role. Furthermore, they suggest that the detailed pattern of arborization of the sensory axons is also guided by prespecified pathways of only ephemeral availability or recognizability and by fasciculation of heteroneuronal processes.

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