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

I. Origin of Natural Variations in the Branching Pattern¹

ANDREW P. KRAMER, JOHN R. GOLDMAN, AND GUNTHER S. STENT²

Department of Molecular Biology, University of California, Berkeley, California 94720

Abstract

The overall sizes, contours, and positions of the receptive fields maintained by different individual cells of the T, P, and N types of mechanosensory neurons in the segmental skin of the leech *Haementeria ghilianii* are not subject to wide variation. However, the locations and contours of the boundaries which separate the various compartments of the sensory field, namely, the major and minor fields, as well as their component subfields, do vary significantly. These variations are reflected in differences in the detailed pattern of arborization of the mechanosensory axon branches that innervate different parts of the receptive field. The appreciable variation in the kinetics of embryonic outgrowth of sensory axon branches, in conjunction with a mechanism of neuronal self-avoidance, is a probable source of this variability in adult receptive field structure. Thus, establishment of these sensory field components would seem to entail a first-come-first-served territorial exclusion between different axon branches extended by the same neuron.

The structure of many invertebrate nervous systems, including that of the leech, is so stereotyped that individual neurons having specific constellations of electrophysiological, morphological, and biochemical properties can be identified in every specimen. The developmental origin and differentiation of adult properties have been examined for various identified neurons in a number of different species, and it has been found that the stereotypy of the mature cell reflects a largely invariant pattern of development. However, for any identified neuron there is always some level of scrutiny at which one can observe individual structural variations (Macagno et al., 1973; Ward et al., 1975; Levinthal et al., 1976; Goodman, 1978; Goodman et al., 1979). The structure of the mature neuron reflects the pattern of its outgrowth during morphogenesis, and individual variations can be construed as developmental noise which arises from elements of its morphogenesis that are not rigidly constrained. In this paper, we examine individual variations in the structure of identified mechanosensory neurons of the segmental ganglia of the

leech ventral nerve cord in both embryos and adults, and we attempt to identify the developmental processes that account for these variations.

The ventral nerve cord of the leech consists of 32 segmental ganglia linked via connective nerves, each ganglion being associated with one of the 32 body segments. Sensory and effector neurons extend their axons from the ganglion to the periphery via four paired segmental nerves, designated as AA, MA, DP, and PP (Ort et al., 1974; Kretz et al., 1976). Three types of mechanosensory neurons innervate the skin. The "touch," or T neuron, responds specifically to slight deformation of the skin; the "pressure," or P neuron, responds specifically to moderate skin deformation; and the "nociceptive," or N neuron, responds specifically to intense skin deformation (Nicholls and Baylor, 1968). The segmental ganglion of the glossiphoniid leech *Haementeria ghilianii*, contains three bilateral pairs of T neurons, two pairs of P neurons and one pair of N neurons (Kramer and Goldman, 1981). All of these neurons maintain receptive fields in the ipsilateral skin, with each T and P neuron innervating a different, characteristic dorsoventral territory. Thus, T_D innervates the dorsal skin, T_L the lateral skin, and T_V the ventral skin. P_D mainly innervates dorsal skin but also has a receptive field in the ventral skin, whereas P_V innervates ventral skin exclusively.

The receptive field of each mechanosensory neuron consists of a *major* field, which is innervated by one or more peripheral axon branches that exit from the ganglion containing the cell body, and two *minor* fields. The minor fields are innervated by axon branches which course in the connective nerve and exit to the periphery via the two adjacent ganglia (Yau, 1976). Some major and minor sensory fields are, in turn, composed of distinct subfields, innervated by separate axon branches of the mechanosensory cell coursing in different segmental nerves within the same segment. 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). This is in pronounced contrast to the boundaries of the receptive fields of serially or contralaterally homologous mechanosensory neurons, which overlap considerably. For instance, both minor fields of a given neuron overlap completely with the major fields of the homologous neurons in the skin of the two adjacent segments.

Study of the embryonic development of the morphology and receptive field structure of these mechanosensory neurons has shown that their axons follow a stereotyped pattern of initial outgrowth from the ganglion along specific pathways toward their appropriate skin territories (Kramer and Kuwada, 1983; Kuwada and Kramer, 1983). Upon reaching the target territory, the sensory axons arborize by extending branches along further specific pathways and finally provide dense innervation of the receptive field. It is a striking feature of this process that two different axon branches of a given mechanosensory neuron stop growing upon nearing each other. Thus, each axon branch excludes the other from the territory it

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² To whom correspondence should be addressed.

Figure 1. Receptive field structure of a P_V mechanosensory neuron. The receptive field is projected on a schematic representation of the skin of three segments on one side of the leech, from the ventral midline (VM) across the lateral edge (LE) to the dorsal midline (DM). The line drawn longitudinally between LE and VM represents the landmark provided by a distinctive narrow band of pigmentation. In each mid-body segment of *Haementeria*, the dorsal skin is divided into three and the ventral skin into five annuli. The dorsal sector of the central annulus (C) in the middle of each segment contains sensory sensillae (open circles). The overall receptive field border is drawn in bold outline. The ventral midline was always cut in these mapping experiments, so that portions of the field that cross it could not be mapped. Shaded and stippled regions together constitute the major field; cross-hatched regions represent the minor fields. The shaded region of the major field is the subfield established by the primary axon (MA) and the stippled region is the subfield established by the secondary axon (PP). Anterior is to the left. Representations of receptive field structure in all other figures conform to the conventions used here.

already occupies. No such growth arrest occurs, however, upon the approach of axon branches belonging to different, albeit contralaterally or serially homologous neurons. These findings have led to the proposal that the nonoverlap of major and minor fields, as well as of their component subfields, is the consequence of a developmental process of neuronal self-avoidance (Kramer, 1982; Kramer and Kuwada, 1983). According to this proposal, axon branches that belong to the same cell (called *isoneuronal* branches) recognize each other as such and do not grow along or across each other during embryonic development.

The work to be reported here consists of a study of the natural variations encountered in the structure of mechanosensory fields of the skin of adult specimens of *H. ghilianii* and in the timing of outgrowth of mechanosensory axons in their embryos. This study shows that, despite the otherwise stereotyped outgrowth pattern of these axons, there is at least one morphological property of the mechanosensory neuron which is subject to appreciable developmental noise: the location and contours of the boundaries which separate major and minor fields, as well as their component subfields, vary significantly from cell to cell. The appreciable variation in the rate of embryonic outgrowth of different sensory axon branches to be reported here is a probable source of this variability in adult receptive field structure. In conjunction with the mechanism of neuronal self-avoidance, it entails a first-come-first-served territorial exclusion between isoneuronal axon branches.

Materials and Methods

Specimens. Adult and embryonic specimens of the glossiphoniid leech *Haementeria ghilianii* were supplied by laboratory breeding colonies main-

Figure 2. Nonoverlapping boundary between receptive subfields in the major field of two P mechanosensory neurons. *a*, Experimental arrangement. The cell body of a neuron was impaled with a microelectrode and an *en passant* suction electrode was attached to each of the MA and PP peripheral nerves that contained one of the two subfield axons whose field was to be mapped. To block conduction of impulses from one subfield axon to the other through the central processes of the neuron, the central processes were tonically hyperpolarized by passage of current into the cell body (*hyp*). Under this condition, impulses are recorded from a subfield axon only when a site on the skin within its receptive field is stimulated. *b*, Boundaries of adjacent receptive subfields determined by focal electrical stimulations of the skin. Squares and triangles on the skin schematic diagram are stimulation sites identified as being in the MA or PP subfields, respectively. Sites at which stimulation produced no nerve impulses are marked by X's. Left, Boundary between MA and PP, major subfields of a P_D neuron. Right, Boundary between MA and PP major subfields of a P_V neuron.

tained at the University of California in Berkeley and in San Diego (Sawyer et al., 1981). Adult specimens were sexually immature animals that weighed 0.5 to 1.0 gm. Embryos were collected and cared for as previously described (Kuwada and Kramer, 1983). Embryos are staged according to a set of external morphological characteristics correlated with each day of development at 27°C (Fernandez, 1980; Weisblat et al., 1980; Stent et al., 1982). Eleven main stages of development are distinguished, and each main stage is divided into fractional stages based on the proportion of the total duration of the stage that has been completed by an embryo. For instance, stage 10(2/5) refers to an embryo that has completed 2 of the total 5 days of stage 10.

Dissections and electrophysiological recordings. Embryonic and adult specimens were dissected and examined with the electrophysiological apparatus as previously described (Kramer and Goldman, 1981; Kuwada and Kramer, 1983).

Mapping the sensory neuron receptive fields. The receptive field of a sensory neuron was mapped on the skin by exciting its sensory endings with focal electrical stimulation and recording the response with an intracellular electrode as previously described (Kramer and Goldman, 1981). A receptive field is defined as that area of skin in which stimulation evokes impulses in the sensory cell in the absence of any apparent synaptic drive. The subfield established by particular peripheral axon branches of a sensory neuron was determined either by cutting all other subfield axons before mapping it or by mapping the whole field first, then cutting the axon branch, remapping, and determining the subfield of the cut axon by subtraction of the second map from the first. The second method is possible because the receptive subfields are nonoverlapping. For ease of presentation and comparison of results from different mappings, the experimentally observed receptive fields were normalized by projecting them onto a standardized schematic drawing of the leech skin, which accurately represents the proportional topography of the relevant skin landmarks. To obtain a statistical measure of the variation in the receptive field configuration of a given type of neuron, replicate receptive field maps were surveyed for a series of homologous neurons in the same and in different specimens. Each receptive

Figure 3. Variability of the overall receptive field boundary of some mechanosensory neurons. Each field was mapped for several neurons. Replicate mappings of each subfield are represented by three receptive field contour lines. The outermost contour line is the smallest which encloses all measured receptive fields and the innermost is the largest which encloses none of them. Half of the field perimeters lie outside and half lie inside the **bold middle contour line**. Thus the **bold line** is the median receptive field boundary of the replicate maps and the **stippled area** is the range of perimeter locations in the sample. *a*, T_D neuron (sample of 7 neurons); *b*, T_V neuron (sample of 10 neurons); *c*, N neuron (sample of 10 neurons; minor fields were not mapped in this sample).

Figure 4. Variability of P_V neuron receptive subfield boundaries. Replicate mappings are summarized schematically as in Figure 3. *a*, Overall major and secondary (PP) subfield boundary variations in a sample of five P_V neurons. *b*, Anterior (left) and posterior (right) minor subfield boundary variations in a sample of seven P_V neurons.

field so obtained was represented by its perimeter, and the normalized perimeters of all of the fields were superimposed on a single skin schematic diagram. The topographical divergence of these perimeters was taken as a measure of receptive field variation. For instance, the range of variation is given by the distance between the smallest outermost contour line that encloses all other perimeters and the largest innermost contour line that encloses none of them. The median perimeter is represented by that contour line for which half the perimeters lie outside and half inside.

Cell staining. Neurons were filled with Lucifer Yellow dye by iontophoretic intracellular injections with microelectrodes containing 5% Lucifer Yellow dye in distilled water. For adult neurons, dye was injected with a steady 5-nA hyperpolarizing current for 10 min. For embryonic neurons, dye was injected with a current of 0.2 to 1.0 nA for 1 to 2 min. Adult ganglia were fixed and examined in whole mount, as previously described (Kramer and Goldman, 1981). Embryonic neurons were examined in the living preparation. Some adult neurons were filled and stained with horseradish peroxidase, using a previously described procedure (Kuwada and Kramer, 1983).

Results

Division of receptive fields into nonoverlapping subfields

The receptive fields of the T , P , and N mechanosensory neurons all share at least two features. They are located on the same side as the cell body and extend over the skin of three adjacent body segments (Nicholls and Baylor, 1968; Yau, 1976; Kramer and Goldman, 1981). The typical subdivision of the receptive field is exemplified by the P_V neuron, shown in Figure 1. The major field is composed of two subfields, of which the larger is innervated by an axon branch in the MA nerve and the smaller by an axon branch in the PP nerve. Such subfields occur also in the minor fields of the P_V cell. The major and minor fields of the P_D and N cells contain subfields as well, formed by axon branches extended via all of the ipsilateral segmental nerves, AA, MA, DP, and PP. (The arrangement of the roots of these four segmental nerves can be seen in Fig. 9.) No subfields have thus far been detected in the receptive fields of any of the three T cells (cf. Table I).

The data presented in Figure 2 show the nonoverlap across the boundary of adjacent subfields, one innervated by an MA axon branch and the other by a PP axon branch, in the major field of a P_V and of a P_D neuron. Thus, the previously demonstrated nonoverlap between isoneuronal major and minor fields holds also for the boundaries between their subfields.

Variability of the subfield configuration of a receptive field boundary

In view of the finding that the embryonic outgrowth of mechanosensory axons is directed initially to particular skin territories (Kuwada and Kramer, 1983), it would be expected that the structure of the receptive fields of each type of mechanosensory neuron is not subject to wide variations. In order to test this expectation, a study of replicate receptive field maps was carried out for each type of neuron. These replicative mappings include the fields of serial and

Figure 5. Extreme variation in MA subfield structure in P_D neurons. (The dorsal domain of the P_D major field, corresponding to the DP subfield, was not mapped here.) a, Receptive subfields of a neuron with MA (shaded), PP (stippled), and AA (lined) subfields. b, Receptive subfields of a neuron with only MA and PP subfields lacking an AA subfield.

contralateral homologues of a given neuron in the same specimen, and of corresponding homologues in different specimens. Figure 3 presents the results of a series of replicative mappings of the boundaries of the fields of T_D , T_V , and N neurons. In agreement with expectation, there are only relatively small variations in the overall size, contour, and position of each type of field.

The expectation of low variability of receptive field structure is not met, however, when replicative mappings of minor fields and of subfields of major and minor fields are carried out. Figure 4 shows the results of such a series of mappings of the receptive field of the P_V neuron. Whereas, in agreement with the results shown in Figure 3, the overall structure of the major field shows only relatively small variations, the size and contours of the minor fields and of the PP subfield of the major field are subject to much larger (relative) variations. Figure 5 shows a case of extreme variation in subfield structure in the ventral domain of the major field of two P_D neurons. For one of these neurons the ventral domain consists of the usual three AA, MA, and PP subfields, but for the other neuron the AA subfield is entirely missing, and the MA subfield occupies the normal AA subfield territory. Similarly, extreme variations in subfield structure are manifest in the ventral domain of the major field of the N neuron as shown in Figure 6. Both PP and MA subfields vary widely in size and contour outline, and in the four cases shown where the PP subfield is entirely missing, the MA subfield occupies part of the normal PP subfield territory. (Another part of the missing PP subfield is occupied by the AA subfield, which was not mapped in this survey.)

Variability of timing of axon extension during development

The substantial variability in the extent, and even in the very presence, of particular receptive subfields indicates that their boundaries are not predetermined by fixed external topographic cues or landmarks. Rather, the observation of axonal self-avoidance during embryogenesis (Kramer and Kuwada 1983) suggests that the variable subfield structure is the result of a first-come-first-served occupation of skin territories by competing isoneuronal axon branches

Figure 6. Variability of boundaries of N neuron subfields in the ventral domain of the major field. Replicate mappings are summarized schematically as in Figure 3. a, PP subfield boundary variations in a sample of nine neurons. b, MA subfield boundary variations in a sample of nine neurons. c, MA subfield boundary variations in a sample of four neurons that had no PP subfield.

and, hence, that the variability of individual subfield contours might arise from a variability in the timing with which different axon branches begin to occupy their peripheral territories during development. This possibility was tested by ascertaining the degree of variability with which the PP subfield axon branch of the P_V neuron is actually extended into the periphery.

The P_V cell body can be identified in the ganglia of dissected embryos just as it begins to extend processes, and the development of these processes can be followed by intracellular injection of the cell body with Lucifer Yellow dye at successive stages of development (Kuwada and Kramer, 1983). Such studies show that at stage 9(2/4), the P_V cell extends a process from the medial pole of the cell body. This central axon forms a T branch in the neuropil, whose arms extend to form the intersegmental axons in the connective nerve. At stage 9(3/4), another process is extended from the lateral pole of the P_V cell body, which becomes the first, or *primary*, peripheral axon branch. This axon will establish the MA subfield of the adult skin. At some time from stage 10(0/5) onward, another, or *secondary* peripheral axon branch is extended from the posterior arm of the central axon; this branch will establish the PP subfield of the adult skin (Fig. 7). The exact starting time or rate of PP axon branch outgrowth is quite variable by comparison, and different P_V neurons in the midbody ganglia of the same embryo can have secondary peripheral axon branches of markedly different length

Figure 7. Fluorescent photomicrograph of left and right P_V neurons filled with Lucifer Yellow dye in a midbody ganglion of a whole mounted, late stage 10 embryo. Anterior is to the left. Both neurons extend a primary (MA subfield) axon from the cell body into the periphery (to the top or bottom) and they extend anterior and posterior central axons in the connective nerves. The left P_V cell (top) extends a secondary (PP subfield) axon out of the ganglion into the periphery where it has arborized (not shown). The right P_V cell (bottom) extends only a short secondary (PP) axon that has not yet entered the periphery.

(Fig. 7). Some of these neurons may have a very short PP axon branch, which has not yet exited from the ganglion, whereas others may have a very long PP axon branch that has grown far into the periphery and begun to arborize in the body wall.

To determine the time span over which PP axon branches first enter the periphery, we examined P_V neurons in embryos at successive stages of development and observed for each neuron whether its PP axon branch was present, and if so, whether it had exited from the ganglion into the periphery, i.e., was "ganglionic" or "peripheral." The percentage of P_V neurons in embryos at a given stage that have ganglionic or peripheral PP axon branches is shown in Figure 8. Outgrowth of the PP axon branches is spread over at least a 6-day period. At the beginning of stage 10, PP axon branches are still mainly ganglionic. By the beginning of stage 11, most of them have become peripheral. (As discussed below, only about 30% of adult P_V neurons have a peripheral PP axon branch at all.) Meanwhile, the MA axon branch of the P_V neuron, whose extension was initiated earlier at stage 9(3/4), is expanding its arborization in the peripheral target territory (Kramer and Kuwada, 1983). Thus, some (secondary)

Figure 8. Developmental time course of PP axon branch extension of the P_V neuron into the periphery. A sample of 5 to 20 (average, 14) P_V neurons were examined in each embryo to determine the percentage of neurons that had extended a PP branch that (1) was confined to the ganglion (i.e., ganglionic axon) or (2) was arborizing in the periphery (i.e., peripheral axon). The heights of bars in the histogram represent the average percentage of ganglionic (hatched bars) and peripheral (solid bars) axons present in embryos at successive stages. The average values and standard deviations assigned to each bar are based on a sample of four to five embryos in a given stage interval. Beginning at stage 10(0–5), each bar is a 2-day stage interval, except the last one, which is a 3-day interval. The horizontal dashed line at 31% indicates the adult percentage of P_V neurons with a PP axon branch.

TABLE 1

Percentage of mechanosensory neurons with functional subfield axons in various peripheral nerves

In most cases, the absence of a subfield, as determined by electrophysiological mapping experiments, was correlated with the absence of the corresponding axon branch, as determined anatomically. However, sometimes an axon branch was apparent anatomically, but no corresponding receptive field was found. Only those cases where anatomical and receptive field mapping results are in agreement are included in the table. The number of such cases is shown in parentheses. These data pertain only to major fields.

Neuron	Peripheral Nerve			
	MA	DP	AA	PP
T_D	0 (5)	100 (7)	0 (5)	0 (5)
T_L	100 (8)	0 (7)	0 (6)	0 (7)
T_V	100 (8)	0 (6)	0 (5)	0 (10)
P_D	100 (19)	100 (17) ^a	64 (11)	60 (10)
P_V	100 (23) ^a	0 (11)	0 (8)	31 (16)
N	100 (14) ^a	100 (9) ^a	23 (13)	89 (9)

^a Primary axons; for N, it is not yet known whether MA or DP is primary.

PP axon branches reach the periphery early in development, when the isoneuronal (primary) MA axon branch as yet occupies little territory and should therefore be able to occupy a substantial territory. Other PP axon branches reach the periphery only late in development, by which time there has already occurred an extensive MA axon branch arborization, and should therefore be excluded from most of their potential target territory. This inference is also supported by the earlier finding that in late stage 11 embryos, some

of the PP axon branches of the P_V neuron occupy large and others small subfield territories relative to the contemporaneous MA axon branch territory, whereas the subfields of those PP axon branches that managed to reach the periphery in mid-stage 10 embryos are mostly large relative to their contemporaneous MA subfield (Kramer and Kuwada, 1983). These findings therefore support the idea that variation in the size of the adult PP subfield arises from variations in the time at which the PP axon branch reaches its peripheral target.

Variability of the number of peripheral axon branches

The broad time span over which subfield axon branches first enter the periphery suggests that some subfield axon branches may enter the periphery so late that all of the potential target territory is already occupied by other isoneuronal axon branches. In this event, a late-arriving axon branch would fail to innervate any skin at all and might be withdrawn, causing the neuron to lack that particular subfield. Figures 5 and 6 show that such variation in the presence of subfield axon branches does, in fact, occur. To quantitate the extent of this variation, we determined by receptive field mapping which subfields of a neuron were present and which were absent, and then confirmed anatomically the presence or absence of the corresponding subfield axon branches. Table I presents for each type of mechanosensory neuron the percentage of neurons for which a given functional subfield axon was observed.³ The results indicate that, in any given type of mechanosensory neuron, some subfield axon branches are invariably present. We designate these branches *regular*. Other branches may or may not be present, and these we designate *variable*. Apparently the three types of T neurons have only a single, regular branch and no variable branch; i.e., their major receptive field has no subfield at all. Thus, variable subfield branches are encountered only with the P and N neurons. Some of the morphological variations of the P_D neuron are illustrated in Figure 9. Not only are there variations in the number of subfield axon branches, but there are also variations in the sites at which the branches originate. For instance, the PP branch of the P_D neuron can arise either off the MA branch in the ganglion (Fig. 9A) or off the DP branch in the periphery (Fig. 9B).

In accord with the proposal that the variability in the number of subfield axons derives from isoneuronal exclusion of latecomers, all of the regular axon branches of the mechanosensory neurons are also developmentally primary axon branches, which means that they are the first peripheral processes to be extended by the neuron and to reach the target skin. Conversely, all of the variable branches are also developmentally secondary branches, which means that they reach the skin only after the primary branches have begun to arborize. Hence, in accord with the proposal of neuronal self-avoidance, the secondary axon branches are variable and the primary axon branches are regular because the former are at a disadvantage compared to the latter in the competition for peripheral territory. (In the case of the P_D neuron, the MA secondary axon branch is a regular branch, but it is the first axon to be extended into the ventral skin territory and thus would not compete with the DP primary axon branch, which grows into dorsal skin territory; however, later-growing AA and PP secondary branches compete with the MA branch in ventral skin and, therefore, are variable branches.)

The data of Figure 8 are also compatible with the interpretation that perhaps as many as 50% of all P_V neurons never extend a PP axon branch out of the ganglion. If this were actually the case, then some of the variability in the presence of this subfield axon branch

Figure 9. Morphological variations of subfield axon branch points in the P_D neuron. These are drawings of the central processes in the segmental ganglion of adult neurons that were stained with horseradish peroxidase. The cell body is closest to the primary DP subfield axon branch and to the central axon branch that bifurcates and extends longitudinally to adjacent ganglia in the connective nerves. From the central branch, neuropilar processes that make synaptic contact with other neurons (Muller and McMahan, 1976) are extended medially, and AA, MA, or PP secondary subfield axon branches are extended laterally into the peripheral nerves. Unlike the other peripheral nerves which emerge from the ganglion in separate roots, the DP and PP nerves separate in the periphery from a common posterior root. The variable subfield axon branches (AA and PP) also have variable sites of origin on the central branch. A, Neuron with four subfield axon branches. The PP branch originates as a branch of the MA branch (at *thin arrow*). The AA branch (at *thick arrow*) and the MA branch originate at separate sites on the central branch. B, Neuron with four subfield branches. The PP branch originates as a branch of the DP branch, but a distinctive neuropilar branch also extends from its other possible site of origin on the MA branch (at *thin arrow*). The AA branch originates as a branch of the MA branch, which also extends a distinctive neuropilar branch to the other possible site of AA branch origin on the central branch (at *thick arrow*). C, A neuron with only two subfield branches. PP and AA subfield branches are missing, but the MA branch extends a distinctive neuropilar branch to the PP branch origin site (at *thin arrow*) and from its AA origin site to the other AA origination site on the central branch (*thick arrow*).

³ The exact frequency of occurrence of the subfield axon branches that display variability was found to differ among leeches from different breeding colonies (e.g., see Table II) and even from the same colony after an interval of several years. With the exception of some data in Table II, all of the results of this paper are based on observations on adult leeches and embryos from the Berkeley colony made during a continuous period.

in the adult skin might be due also to a variability in factors that act on its initial outgrowth, rather than being wholly the consequence of isoneuronal exclusion in the periphery.

If it is indeed the case that variable axon branches are sometimes absent because, even having been initially extended into the periphery, they arrived too late to compete with the regular branches and were retracted, then all of the neurons would initially extend this variable branch. One would therefore expect that a higher percentage of neurons extend the variable branch during development than retain it to adulthood. The data presented in the histograms of Figure 8 are consistent with this expectation, although they do not really provide statistically significant support for it. But they suggest, at least, that by early stage 11 about 60% of the embryonic P_V neurons have extended a variable PP axon branch, ganglionic as well as peripheral, compared to the approximately 30% that possess a PP subfield in adulthood (cf. Table I). Therefore, some of the initially formed PP axon branches do appear to be retracted eventually. Moreover, the finding presented in the companion paper (Kramer and Stent, 1985), that the percentage of P_V neurons that extend a secondary PP axon branch can be increased from 30% to nearly 100% by cutting the primary MA branch, supports the inference that, normally, all of them extend at least a tentative PP branch.

Pattern of variability. To probe the level at which the source of the variability in axon branching is located, we ascertained whether there is any covariation at the ganglionic, cellular, or individual axon branch level. For this purpose, we surveyed the pattern of variation of the variable subfield branches of the P_D , P_V , and N neurons in adult specimens of *H. ghilianii*. First, we examined the patterns to see whether the two variable AA and PP subfield branches of the P_D neuron are correlated at the single cell level. This survey showed that, in individual P_D neurons, combinations of AA and PP subfield branches occur with a frequency about equal to that expected if the presence of an AA or PP variable branch were determined independently (Table II). Thus, the variability does not seem to be controlled at the level of the whole cell. Furthermore, the average frequency of occurrence of the AA branch is different in animals raised in different breeding colonies, where the conditions may not have been exactly comparable. This would suggest that the chance of forming variable subfield branches can be influenced by genetic or environmental factors.

Second, we examined embryos to see whether in essentially mature, stage 11 embryos the presence or absence of the variable PP branch of the P_V neuron is correlated: (1) in the two paired cells on the right and left sides of the same ganglion, (2) in the (isoneu-

ronal) major and minor field branches of the same cell, and (3) in the (heteroneuronal) major and minor field branches of the two serially homologous neurons that exit from the same hemiganglion. Figure 10 presents the outcome of a typical examination. In this specimen, of two paired P_V neurons in the same ganglion, the left cell did and the right cell did not extend a variable PP axon branch in establishing the major field. A third P_V neuron, whose cell body was located on the left side of the next posterior ganglion, did not extend a variable PP axon branch into the major field of its own ganglion but did extend one into its minor field on the left side of the anterior ganglion in which the other two P_V cells were located. Thus, both major and minor fields in the left skin of the anterior segment were innervated by a variable PP branch, whereas the major fields in the right skin of the anterior and the left skin of the posterior segments lacked innervation by a variable PP axon branch. The results of this survey are summarized in Table III. Evidently, there is no significant correlation in the occurrence of the variable axon branch of P_V neurons in right and left sides of the same ganglion or in the major and minor field axons of the same neuron. This latter result means that the formation of the variable branch is not the expression of an intrinsic property of the individual cell. However, there is a statistically significant correlation in the occurrence of variable axon branches in major and minor field processes extended by two different P_V cells from the same hemiganglion. The presence of a variable axon branch of the minor field processes, therefore, is strongly correlated with the presence of a variable axon branch of the major field processes on the same side (15 of 15 instances), although the reverse situation is only weakly correlated (15 of 61 instances). Thus, formation of the variable PP axon branch appears to be governed by a variable factor whose range of action is restricted to one side of a single segment. Although this factor increases the probability of variable axon branch outgrowth of all P_V cell processes in a hemiganglion, it affects outgrowth of the major field axon branches more strongly.

Discussion

What is constant and what is variable. The results presented here show that the structure of the receptive field of leech mechanosensory neurons has both constant and variable features. Thus, the location, size, and contour of the overall field of a given type of mechanosensory neuron are quite constant. This constancy occurs because, from the very outset of the development of the receptive field, the axon branches grow directly to the appropriate peripheral territory along prespecified pathways that are occupied by the axon branches in a regular temporal order (Kramer and Kuwada, 1983; A. P. Kramer and D. K. Stuart, manuscript in preparation). Moreover, this stereotyped spatiotemporal growth pattern not only gives rise to the constant global properties of the receptive field, but it also leads to a pattern of sensory axon arborization which is sufficiently stereotyped to permit the identification and naming of particular first and second, and even higher, order axon branches. Nevertheless, there is a significant amount of variation in the arborization pattern; indeed, it is likely that no two homologous mechanosensory neurons develop an identical pattern of arborization of their peripheral axons.

It would appear that the constant features of the axonal arborization pattern take their origin in a constant grid of possible growth pathways which guide the mechanosensory axon branches to their peripheral target territories (Kramer and Kuwada, 1983; A. P. Kramer and D. K. Stuart, manuscript in preparation). As for the variable features of that pattern, i.e., its developmental noise, they seem to derive from the need of the growth pathways to be occupied in a fixed order by the growing branching system. Thus, as discussed further below, there are variations in the timing of outgrowth to the periphery of the axon branches that establish different parts of the receptive fields, and these variations in turn lead to variations in the exact manner in which the growing axon branch system follows the available pathways and manages to occupy particular skin territories. Thus, one key feature of a mechanosensory neuron's identity and

TABLE II
Frequency of occurrence of combinations of AA and PP subfield axons on individual P_D neurons compared to frequency expected by chance combination

Specimen	Frequency (Percentage)			
	AA and PP	AA only	PP only	Neither
Berkeley leeches ^a (n = 10)	40 (42)	30 (28)	20 (18)	10 (12)
San Diego leeches ^b (n = 23)	22 (18)	9 (12)	39 (43)	30 (27)
Combined ^c (n = 33)	27 (26)	15 (16)	33 (35)	24 (23)

^a Observations were made on adult specimens raised in the Berkeley colony. Data are from P_D neurons also entered in Table I. Expected frequencies (numbers in parentheses) are based on a 70% frequency of the AA branch and a 60% frequency of the PP branch.

^b Observations were made on adult specimens raised in the San Diego colony. Data are only from anatomical observations on P_D neurons stained by horseradish peroxidase. Expected frequencies are based on a 30% frequency of the AA branch and a 61% frequency of the PP branch.

^c Expected frequencies are based on a 42% frequency of the AA branch and a 61% frequency of the PP branch.

Figure 10. Developmental variations of PP axon branch extension from P_V neurons. A, Two P_V neurons on opposite sides of two adjacent ganglia of an early stage 11 embryo were filled with Lucifer Yellow dye and photographed in whole mount from the ventral aspect. Anterior is to the left. The left (upper) neuron does not have a PP axon branch in the ganglion containing its cell body but does extend a PP axon branch in the anterior adjacent ganglion. The right (lower) neuron does not extend a PP axon branch in either ganglion. B, The P_V neuron located on the left (upper) side of the anterior ganglion was then filled with Lucifer Yellow dye and the preparation was rephotographed. That third P_V neuron does extend a PP axon branch.

TABLE III

Associations of occurrence of variable PP subfield axon branches in the P_V neuron in stage 11 embryos

The data of this table lead to the following conclusions. For Row I, the frequency of variable branches (138 of 258 = 0.53) is about equal for right (R) and left (L) ($p < 0.28$). Moreover, there is no significant positive or negative correlation of joint formation of right and left variable branches in the same ganglion, as indicated by the agreement of the data with expected random association values ($p < 0.9$). For Row II, the frequency of major variable branches (74 of 137 = 0.54) is about twice that of minor variable branches (36 of 137 = 0.26) ($p < 0.001$). There is no significant positive or negative correlation of joint formation of major and minor variable branches by the same neuron, as indicated by the agreement of the data with the expected random association values ($p < 0.27$). For Row III, there is a significant positive correlation between the formation of major variable branches by one neuron and of minor variable branches by another neuron in the same hemiganglion, since the numbers of "Both" and "Neither" cases are significantly higher and the numbers of "Ma only" and "Mi only" cases are significantly lower than the expected random association values ($p < 0.016$). The p values shown are based on χ^2 tests of these data for the random association hypothesis.

Row	Comparison	Number of Variable Branches Found					N_1^c	N_2^d
		Both	R only or Ma only ^a	L only or Mi only ^b	Neither			
I	R & L in same ganglion	38 (37) ^e	37 (32)	25 (32)	29 (28)	138	258	
II	Ma & Mi field of same neuron	15 (19)	59 (55)	21 (17)	42 (46)	110	274	
III	Ma & Mi field in same hemiganglion	15 (9)	46 (52)	0 (6)	43 (37)	76	208	

^a R, right; Ma, major.

^b L, left; Mi, minor.

^c N_1 , total number of variable branches found.

^d N_2 , total number of fields surveyed.

^e Numbers in parentheses, values expected from random association between right and left variable branches (Row I), and between major and minor variable branches of the same neuron (Row II), or between major and minor variable branches of two homologous neurons in the same hemiganglion (Row III).

function, namely, the location of its entire receptive field, is specified in a stereotypic fashion by the guidance cues which shape its growth, whereas the exact details of its axonal arborization pattern, apparently less important functionally, are more or less unspecified and subject to variation. The extent of this variation would be amplified by the intervention of self-avoidance, which would prevent two isoneuronal axon branches from following the same route of the prespecified pathway system.

Source of the natural variation in subfield structure. A direct experimental test of the proposal of self-avoidance of isoneuronal mechanosensory axons in development is provided in the companion paper (Kramer and Stent, 1985). There it is shown that, if the outgrowth of the axon branches which establish the minor field is prevented or delayed experimentally, the major field axon branches spread into the vacated minor field territory. Conversely, interference with the growth of the major field results in the spread of the minor field axon branches into the vacated major field territory. Hence, the natural variation in the number of subfield axons branches of individual P_0 , P_V , and N mechanosensory neurons, and the considerable variation in subfield size reported here, can also be explained in terms of the mutual territorial exclusion of isoneuronal subfield branches by self-avoidance. The source of that variation in receptive field structure would be a variation in the timing of subfield axon branch outgrowth during embryonic development (Fig. 8).

What, in turn, could be responsible for the considerable variation in the time at which a neuron extends an axon branch from the ganglion into the periphery? Pronounced variation in timing of axon branch extension of homologous neurons in the same embryo is not restricted to the leech nervous system, having been observed also in grasshoppers (Raper et al., 1983). As indicated by the data of Table III, the source of the variation in the extension of the PP branch of cell P_V appears to be a variable factor, whose range of action is restricted to one side of a single segment. In view of the evident guidance role of pathways in the outgrowth of axon branches, one plausible candidate for this variable factor is the pathway itself. That is to say, since outgrowth of the PP axon branch of cell P_V into the periphery could not begin until its proximal pathway structure was in place, local (i.e., hemisegmental) variations in the maturation of particular components of the pathway system would lead to the kind of variations in the time of axon extension which, as observed here, are responsible for the natural variability of mechanosensory receptive field structure. Moreover, the present finding that no minor field PP axon branch will grow out unless a heteroneuronal major field PP axon branch is also present in the same peripheral nerve implies either that the major field PP branch is required for growth guidance of the minor field branch or that growth of both major and minor field PP branches is guided by the same (variable) pathway and this pathway often fades away before the minor field branch can begin its outgrowth into the periphery.

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