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Development of a sensory afferent projection in the grasshopper embryo

II. Growth and branching of peripheral sensory axons within the central nervous system

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SUMMARY

The morphogenesis of several types of sensory axon branching patterns has been described by cobalt filling the cercal nerve of the grasshopper embryo at a series of different stages in development, thus staining the earliest sensory axons as they grow through the CNS. This embryonic sensory projection contains all five types of cercal afferents seen in the adult, and no new sensory tracts are added during postembryonic life. When the embryonic sensory axons first follow their pioneer axons into the neuropil they choose pathways which are characteristic of the adult sensory tracts. Since the afferents follow these paths without sending collaterals into the other tracts, it appears that the growing axon chooses its specific pathway without extensive exploration of alternative routes. Likewise, nearly all of the branches which arise from the embryonic sensory axons remain within the eventual domain characteristic of each cell type. This precise, determinate pattern of initial growth implies that the sensory axons are guided through the neuropil and achieve their final branching patterns with a minimum of overgrowth and pruning. The fact that initial growth is so precise also suggests that the parameters which guide the growing axon may help to determine its eventual pattern of synaptic connectivity by limiting its physical access to large portions of the neuropil which contain potentially compatible synaptic partner cells. Two different types of neurons may be supplying the sensory afferents with guidance cues: (i) Although most of the cercal sensory axons diverge from the cercal pioneer axons within the CNS, some sensory afferents continue to follow the pioneers through several ganglia. (ii) In the adult, a large number of the cercal sensory axons form a hollow shell of arborization around the main dendrite of an identified synaptic target cell, the Medial Giant Interneuron (MGI). This structure, the interneuron dendrite and the shell of sensory arbor, is called the cercal glomerulus. Since the MGI's dendrite is already present at the stage when the first sensory axons enter the CNS, interactions between these cells could serve to guide the glomerular sensory axons away from the pioneers into their future tracts.

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INTRODUCTION

A neuron's branching pattern shapes the integration of its electrical signals (Rall, 1964) and reflects the spatial arrangement of its synaptic contacts with other cells. As a result, the developmental events which underlie the growth and branching of neuronal processes play a fundamental role in shaping the nervous system's anatomical and functional organization. The study of nervous system development would benefit greatly from a clear understanding of how neurons acquire their final branching structures. The dynamics of this process have been observed directly by following growing neurons in cell culture (Bray, 1973), and the normal *in vivo* development of specific branching patterns has been reconstructed by selectively staining single identified neurons (Truman & Reiss, 1976; Goodman, O'Shea, McCaman & Spitzer, 1979; Bentley & Toroian-Raymond, 1981) or populations of essentially identical neurons (Ramon y Cajal, 1960) in animals of different ages. In this way the formation of a branching pattern is considered at the level of such precise cellular events as the choice of particular pathways or the appearance and disappearance of specific branches. Events of this kind can then be subjected to experimental analysis in an attempt to determine the parameters which regulate neuronal growth (Kimmel, Shabtach & Kimmel, 1977; Goodman & Ridge, 1980). The present paper describes the development of sensory axon branching patterns in the embryo of the grasshopper *Schistocera nitens* towards this end. It was found that most of the sensory axons grow directly to their eventual sites of aborization and do not require extensive pruning of excess branches to achieve their final structure. This suggests that the growing axon experiences contact with a restricted set of the available target cells, and that the initial pattern of axonal growth could be a determinant for the patterning of synaptic connexions.

The cercal sensory system was chosen for this investigation because of the considerable amount of background knowledge from a variety of insect species. The cerci are abdominal sensory appendages whose neurons project into the terminal abdominal ganglion of the ventral nerve cord. Many of these cercal neurons are mechanosensory, and their axons synapse with a set of large, individually identifiable cells collectively known as the Giant Interneurons (Murphey, Mendenhall, Palka & Edwards, 1975). These interneurons project through the abdominal nerve cord into the thorax, where they exert an excitatory synaptic influence upon the motor neurons of the legs (Ritzmann & Camhi, 1978), and are believed to mediate the animal's evasion response to mechanical disturbance of its hindquarters (Roeder, 1963). Although the synaptic connexion of the cercal sensory neurons and Giant Interneurons has been described in several species of grasshopper and locust (Cook, 1951; Hoyle, 1958; Seabrook, 1971), it has been more thoroughly studied in the cricket (Edwards & Palka, 1974) and cockroach (Westin, Landberg & Camhi, 1977). The body of available knowledge includes: the morphology and physiology of individual sensory neurons

(Palka, Levine & Schubiger, 1977; Murphey, Jacklet & Schuster, 1980); the morphology (Murphey *et al.* 1975) and physiology (Murphey, Palka & Hustert, 1977) of individual interneurons; the synaptic physiology of their connexion (Callec, Guillet, Pichon & Boistel, 1971); and the pattern of connectivity between the sensory neurons of different receptive fields and individual interneurons (Palka & Olberg, 1977; Matsumoto & Murphey, 1977*a*). The postembryonic development of the cercal sensory system has also received attention. Palka & Edwards (1974) amputated the cerci of newly hatched crickets, then allowed these organs to regenerate at a later stage. The sensory axons arising from the regenerate appendages were able to locate the Giant Interneurons and establish appropriate synapses despite the absence of any pre-existing cercus-to-Giant connexion. Transplantation experiments have shown that the sensory neurons can also form seemingly normal central connexions despite ectopic relocations of their peripheral cell bodies (Edwards & Sahota, 1967; Palka & Schubiger, 1975). If cercal regeneration is prevented by repeated extirpation, the resulting sensory deprivation stunts the growth of specific Giant Interneuron dendrites (Murphey *et al.* 1975), and alters the synaptic contribution of remaining sensory cells (Palka & Edwards, 1974; Levine & Murphey, 1980). Silencing the electrical activity of the presynaptic sensory neurons without surgical intervention, either by immobilizing the sensory neuron's transducing element (Matsumoto & Murphey, 1977*b*) or by single gene mutations which cause its loss (Bentley, 1975), has similar morphological and physiological results.

This paper aims to lay a foundation for studying developmental phenomena such as axonal pathfinding and synaptic target cell interaction during embryogenesis. By examining the peripherally located sensory neurons and the centrally located interneurons as their processes grow across the intervening distance, make their initial contacts, and first establish synaptic connexions, it will be possible to describe the primary developmental interactions that occur between these synaptic partner cells. The cercal sensory system of the grasshopper is not as well characterized as that of the cricket or cockroach, but the grasshopper embryo is especially large and has proven accessible to a variety of different techniques in the study of neuronal origins (Bate, 1967*a*; Goodman, Pearson & Spitzer, 1980; Keshishian, 1980) and single cell differentiation (Goodman *et al.* 1979). A companion paper (Shankland, 1981) describes the pre-sensory pioneer neurons (Bate, 1976*b*; Edwards & Chen, 1979) whose axons actually lay down the pathway of the cercal nerve, while the present paper deals with the following stage in development when the first cercal sensory axons grow along this nerve into the embryonic ganglion and establish their central branching patterns. Preliminary observations on the embryogenesis of one particular central target cell, the Medial Giant Interneuron (MGI), have also been included. These results have been summarized elsewhere (Shankland, 1979; 1980).

METHODS

Most of the techniques used here have been described in detail in the preceding paper (Shankland, 1981). The percentage staging system is presented in Bentley, Keshishian, Shankland & Toroian-Raymond (1979).

The central projections of the cercal sensory axons were stained by cobalt filling the cercal nerve. Embryos at a series of different ages were dissected and pinned out while immersed in tissue culture medium. Either the isolated sensory nerve or the whole cercus, which had been torn open to expose the nerve, was then sealed for 3–4 h into a chamber containing 1 % $CoCl_2$, after which the ganglia were bathed in ammonium sulphide to precipitate cobalt ions that had diffused into the axon terminals. The postembryonic sensory projection was stained by cutting away a large portion of the cercus from an immobilized grasshopper and placing a drop of $CoCl_2$ solution on the exposed nerve, then sealing over the drop with a dome of vaseline. Since single sensory axons could not be traced in most of the whole nerve cobalt fills, the cerci of four animals were left intact and a drop of $CoCl_2$ solution placed over a cluster of 10–20 plucked or broken sensory hairs, thereby staining a smaller number of fibres within the CNS. The results presented in this paper consist of the cobalt-filled silver-intensified afferent projections of 29 embryos (ages 65–100 %), 4 first instars, and 16 adults.

The MGI was cobalt filled by transecting the connective between ganglia A_7 and A_6, and placing the cut end into a 3 % $CoCl_2$ solution. The connective was also partially sectioned outside the cobalt reservoir to keep the other Giant Interneurons, whose axons lie in the dorsolateral region of the connective, from being filled as well. The dissected preparation was then maintained for 4 h in tissue-culture medium with the tracheae underlying the abdominal nervous system opened to the atmosphere to ensure that the ganglia received a continuous supply of fresh air. The anatomical description of the MGI presented here is based upon eight silver-intensified cobalt fills.

RESULTS

Anatomy of the cercal afferent projection

The central projections of the cercal sensory axons were stained by cobalt fills of the cercal sensory nerve. These cobalt fills did not stain efferent neurons with central somata, implying that this trunk of the cercal nerve is composed entirely of afferent sensory fibres. This sensory nerve does fuse with motor nerves, as well as other sensory nerves, outside the cercal lumen, and the cercal afferents thus enter the terminal abdominal ganglion as part of the composite cercal nerve root (Seabrook, 1968).

The cercal sensory afferents fall into three general classes on the basis of their projections into the CNS. One class of sensory axons, the glomerular

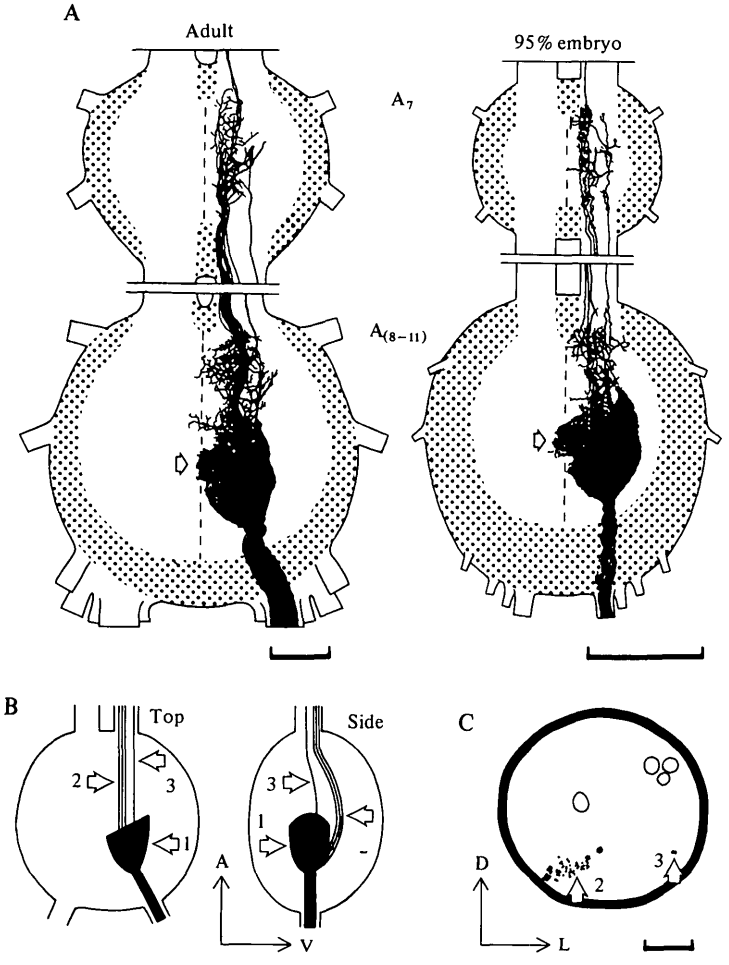


Fig. 1. Projection of the cercal sensory axons into the abdominal CNS. (A) Camera-lucida tracings of silver-intensified cercal nerve cobalt fills from an adult and from an embryo near the time of hatching. There are no obviously novel sensory tracts added to the projection between these two ages. Most of the axons terminate in the cercal glomerulus (arrows) of the fused terminal ganglion, which derives from embryonic abdominal segments A_8-A_{11}, while two other sensory tracts project through the connective into the more anterior, unfused ganglion A_7. The external cell body layer of the ganglia is shown by stippling, and the midline marked by a broken line. Scales 100 μm. (B) Schematic showing the distribution of the different types of cercal sensory axons within the terminal ganglion: 1, G afferents; 2, MV afferents; 3, L afferents. Axes are given for the sideview: A, anterior; V, ventral. (C) Disposition of interganglionic sensory axons in the connective immediately anterior to the terminal ganglion. Camera-lucida tracing from a 6 μm transverse section of a silver-intensified cobalt fill. The sensory axons are labelled as in part B. The four largest fibres in the connective (the ascending axons of the Giant Interneurons) are also shown. Axes: D, dorsal; L, lateral (Adult). Scale 20 μm.

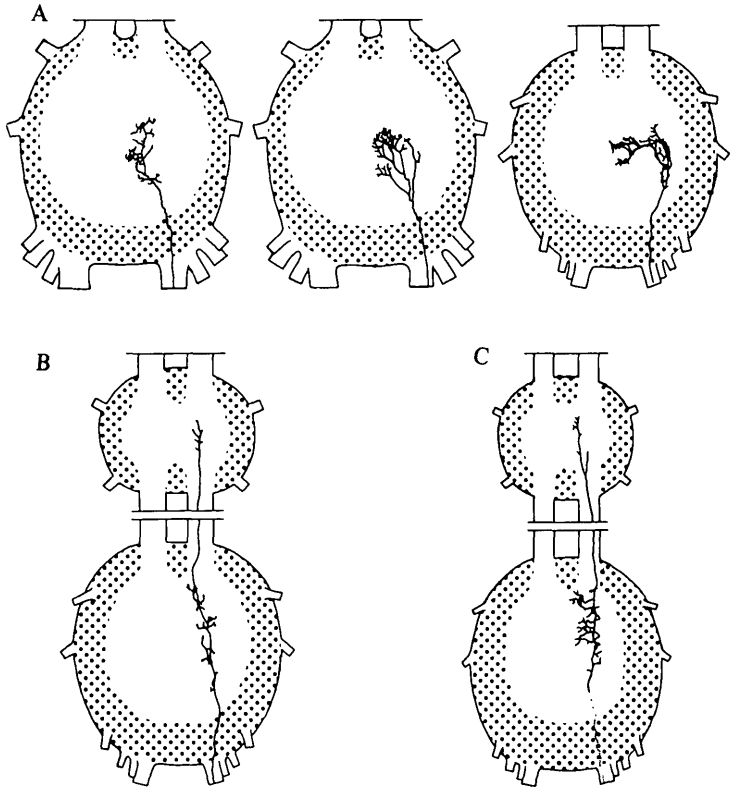


Fig. 2. Central branching patterns of individual sensory axons. Camera-lucida tracings from silver-intensified cobalt fills. (A) G, afferents of the three glomerular tracts. From left to right: ventral tract (Adult); dorsal tract (Adult); lateral tract (First instar). The G afferents terminate in the cercal glomerulus of the terminal ganglion and do not extend into any other ganglia. (B) The MV afferent projects through either two or three ganglia and produces short, diffuse sidebranches. (First instar.). (C) The L afferent projects through the lateral neuropil of two ganglia and has long, medially-directed sidebranches (85% embryo).

(G) afferents, arborize entirely within the terminal ganglion. The other two classes, the lateral (L) and medioventral (MV) afferents, project through the ipsilateral connective into more anterior ganglia. Figure 1 explains the distribution of these different types of sensory axons in a typical cobalt fill. It can be seen that the preponderance of sensory axon arborization is ipsilateral to the nerve of entry, and those branches which do project over the midline extend across no more than 20% of the contralateral neuropil. The branching patterns of single sensory axons are given in Fig. 2.

G Afferents. The majority of sensory axons stained by cercal nerve cobalt fills terminate in a well-defined glomerulus which lies a short distance from the point where the axons enter the neuropil (Fig. 1). This glomerulus is densely packed with fine processes and stains darker than surrounding neuropil with the Rowell silver stain (Fig. 3A). Although the G afferent aborization is

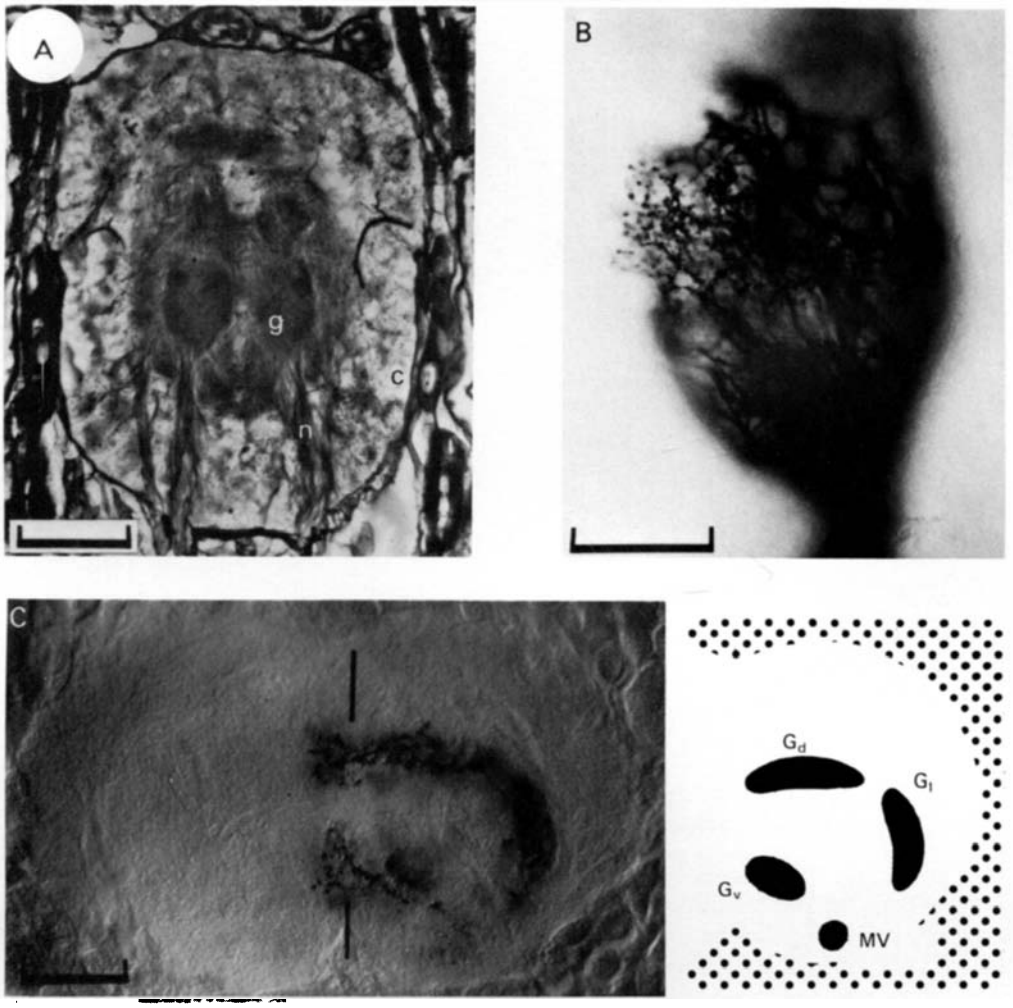


Fig. 3. Cercal glomerus. (A) Photomicrograph of a 10 μm horizontal section of the terminal ganglion stained with the Rowell silver method (Rowell, 1963). The glomerulus (g) stains darker than surrounding neuropil and can be seen to lie at the end of the sensory afferent tract (n) which leads across the cell body cortical layer (c) from the cercal nerve (90% embryo). Scale 50 μm. (B) The glomerular sensory axons form a densely packed shell of heavily ramified and interwoven terminal aborization. Photomicrograph of a silver-intensified cobalt fill in a cleared, whole-mounted ganglion (Adult). Scale 50 μm. (C) The glomerular sensory axons arborize around the surface of the glomerulus forming a hollow shell. Photomicrograph of a 40 μm transverse section from a silver-intensified cobalt fill. Diagram at the right depicts the relative distribution of the different afferent tracts: G_d, dorsal glomerular tract; G_l, lateral glomerular tract; G_v, ventral glomerular tract. Broken line marks the midline. (First instar.) Scale 25 μm.

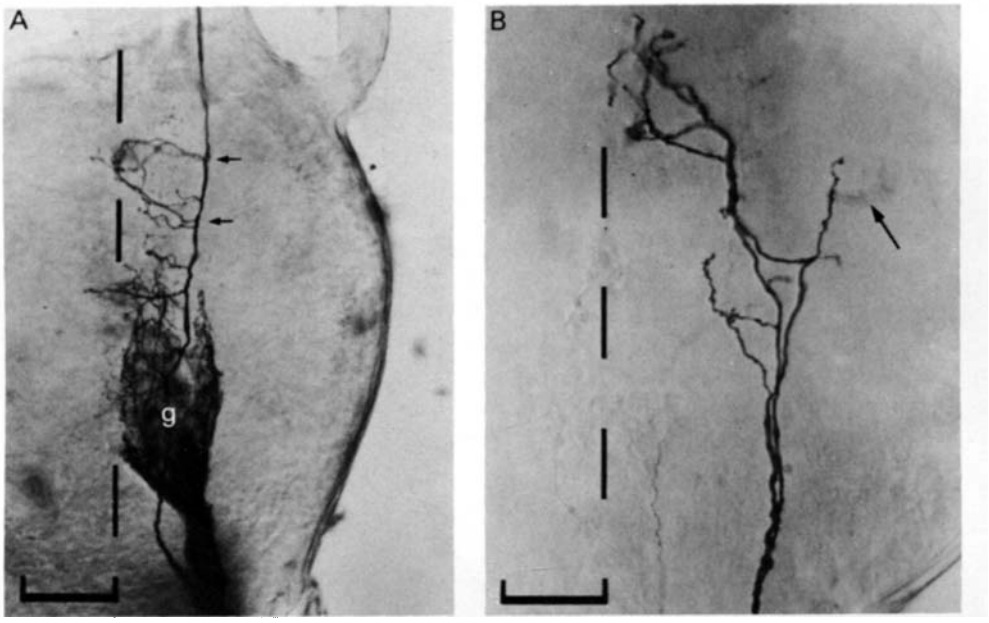


Fig. 4. Branching pattern of the L afferents. Photomicrographs of silver-intensified cobalt fills, seen in whole-mounted ganglia. Broken line marks the midline (Adult). (A) Two L axons projecting through the terminal ganglion and into the connective. These fibres pass across the centre of the glomerulus (g), and give rise to long, medially-directed sidebranches with sparse terminal aborization. Arrows mark the two large sidebranches which were seen on virtually every L axon. Note that the two axons have an almost identical branching pattern. Scale 100 μm. (B) Two L afferents terminating in ganglion A_7. Most of the branches are directed anteriorly toward the midline, but one unbranched process (arrow) dives into the ventral region of the more lateral neuropil. Scale 50 μm.

predominantly ipsilateral to its nerve, the glomeruli on the two sides of the ganglion have a small region of overlap at the midline in that portion of the ganglion which derives from the posterior commissure of embryonic segment A_9. (See Shankland, 1981, for the embryonic segmentation of the terminal ganglion neuropil.) In 13 out of 14 adults, these G axon branches were the most posterior crossing tract of the sensory projection.

The G afferents diverge into three separate tracts at the posterior tip of the glomerulus. Each tract curves anteriorly along the perimeter of this structure, and the constituent axons ramify over its surface. As a result, the afferent arborization forms a lamina (approximately 7 μm thick in the adult) of densely interwoven terminal branches (Fig. 3*B*) which surrounds the remainder of the glomerulus like a shell (Fig. 3C). Figure 2A gives representative branching patterns for single axons of each glomerular tract. (i) The axons of the *ventral glomerular tract* (G_v) pass beneath the medial edge of the glomerulus, then turn dorsally and branch around the anterior side. These axons give rise to many short sidebranches along this route. (ii) The axons of the *dorsal glomerular*

tract (G_d) bifurcate repeatedly over the dorsal curvature of the glomerulus. (iii) The axons of the *lateral glomerular tract* (G_1) follow the lateral margin of the glomerulus and have long, extensively arborized sidebranches which project medially around the circumference of the sensory shell. Most G_1 afferents have sidebranches which follow the dorsal curvature of the glomerulus, but a few axons send their sidebranches around the ventral side. These medial sidebranches pass over the sensory axons of the other two glomerular tracts, and thus join the disparate G afferents into a single, relatively continuous shell of arborization.

The interior of the cercal glomerulus is occupied by the dendrites of the terminal ganglion's Giant Interneurons. One of these cells, the MGI, has an egg-shaped cluster of dendritic branches which precisely fits inside the glomerular sensory shell (Fig. 8A). The other Giant Interneurons could not be selectively backfilled like the MGI, but whole connective cobalt fills show that they also have large dendrites which arborize inside the glomerular sensory shell. Thus, the cercal glomerulus consists of a core of dendritic arborization enwrapped by a layer of sensory axon terminals, and is probably the main site of the cercus-to-Giant synaptic connections that have been reported by physiological techniques.

L Afferents. Cobalt fills of the adult afferent projection stained 1–3 large (1–2 μm dia.) sensory axons which form a bundle running through the centre of the glomerulus (Fig. 4A). These L afferents enter the neuropil at the medial edge of the cercal nerve tract, but veer into a more laterally situated longitudinal tract which they follow, along with the sensory afferents of the ninth dorsal nerve, into the ventrolateral region of the connective (Fig. 1C).

The L sensory axons have a highly stereotyped central branching pattern. They bear long, medioventrally oriented sidebranches within the terminal ganglion (Fig. 2C). Certain of these branches could be identified from one animal to the next. For instance, 12 of the 13 adult L afferents which were traced in full had a pair of sidebranches which crossed the midline in the most anterior portion of the ganglion (Fig. 4A). The more anterior of these two branches follows a direct medial course, while the more posterior branch is slanted anteriorly so that it terminates in the same region of the neuropil. The L sensory axons have other crossing branches whose locations are more variable, with a total of 3–5 crossing branches per axon within the terminal ganglion. However, only 1 out of a total of 27 L afferents that were stained had a branch crossing the midline posterior to the cercal glomerulus.

The L afferents terminate in ganglion A_7, the last unfused abdominal ganglion, and do not project into ganglion A_6. Most of their arborization lies in the anteromedial region of this ganglion, but some of these axons have a single, unbranched process which dives away from the rest into the ventrolateral sector of the neuropil (Fig. 4B).

MV Afferents. This class of sensory axons passes underneath the cercal glomerulus (Fig. 1B) and follows a longitudinal tract (or closely spaced group of tracts) through the medioventral region of the neuropil and connective

(Fig. 1C). Most of these afferents terminate in ganglion A_7, but a few continue anteriorly into ganglion A_6. Unlike the other cercal afferents, they do not have a highly organized arborization but rather give rise to a diffuse network of fine sidebranches which permeate the ventral half of the neuropil on the ipsilateral side (Fig. 1A; 2B). Some of these branches cross the midline in ganglia A_6, A_7, and the anterior region of the terminal ganglion, but none of the MV axons seen here had branches crossing the midline posterior to the cercal glomerulus.

Although there were no appreciable sex-related differences in the cercal afferent branching patterns, the number of MV axons stained by cobalt fills did differ between male and female adults. While all eight fills of adult male grasshoppers stained more than 20 fibres within the MV tract, no more than 11 MV axons were stained in any of the six fills of female adults. Since male and female grasshoppers develop unequal numbers of certain types of sensory receptors in the last instar and adult (Shankland, in preparation), this disparity might reflect an actual difference in the sensory neuron population.

Growth and branching of embryonic sensory axons

The cercal afferent projection of the grasshopper is first established at 40% of embryonic life (Shankland, 1981). A pair of cercal pioneer axons grow from the periphery into the CNS at this stage, and thereby lay the foundations of the cercal nerve. When the epidermal sensory neurons differentiate at 55–60% (Shankland, in preparation), their axons grow to the CNS along this pre-existing pioneer nerve. Since the sensory afferents (identified by their large numbers and characteristic central distribution) were first seen in the terminal ganglion at 65%, it was possible to calculate that these axons are growing at a rate of 10–20 μm/h within the nerve.

The growing sensory axon terminates in an ellipsoidal or spindle-shaped growth cone (Fig. 6A) which resembles the growth cone of the cercal pioneer axon (Shankland, 1981). These growth cones often have a single long (30 μm) terminal filament with filopodia arising from its sides. There are also filopodia covering the sides of the immature axon shaft which create a diffuse halo around the newly formed sensory tracts. These lateral filopodia disappear over the course of embryonic development, leaving the axon shaft smooth and bare.

The sensory axons begin to branch within 5% of embryogenesis after reaching the CNS, forming secondary growth cones which vary greatly both in their shape and number of filopodial extensions. Some axonal growth cones have a forked morphology (Fig. 5A) which suggests that secondary growth cones arise by bifurcation of the axon's growing tip. Sequential bifurcations of this kind most likely account for the multiple growth cones and highly arborized branching pattern of the immature G_d axon seen in Fig. 5B. However, the sequence of events in the formation of specific identified branches of the L sensory axon (Fig. 7B) indicate that branches can also sprout from the side of the main axon shaft after the primary growth cone has passed more than a

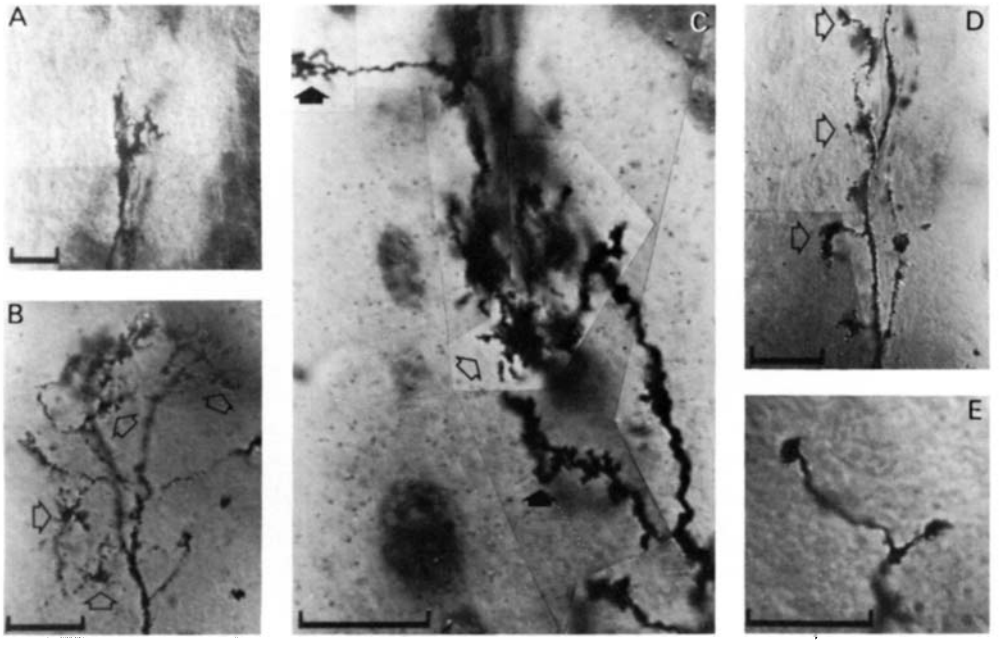


Fig. 5. The growth, branching, and morphological maturation of embryonic sensory axons. Photomicrographs of 10–25 μm sections from silver-intensified cobalt fills. (A) An MV axon terminating in a bifurcated growth cone suggestive of incipient division into separate branches (85% embryo). (B) Ramified G_d axon with growth cones (arrows) at the tips of each branch (80% embryo). (C) MV axons bearing non-terminal, filopodia-covered swellings (hollow arrow) believed to be potential or incipient sidebranch growth cones. This section also contains an L afferent with branches that either terminate in growth cones (upper solid arrow) or are covered with knobby protrusions (lower solid arrow) (85% embryo). (D) Arrows mark swollen, immature sidebranches arising from an MV axon (80% embryo). (E) Smooth terminal swellings presumed to be presynaptic boutons (First instar). Scales 10 μm.

hundred microns further along. Many immature sensory axons have non-terminal, filopodia-covered swellings which may be potential or incipient sidebranch growth cones that have not yet parted from the axon shaft (Fig. 5C). These two alternative forms of branch formation, terminal bifurcation and sidebranch sprouting, have also been observed with time-lapse photography of growing axons in cell cultures (Bray, 1973). The majority of branches were reported to arise from growth cone bifurcations, but in some instances a growth cone would leave in its wake a patch of ruffling membrane that became a sidebranch growth cone after a short delay.

Some of the axonal branches stained at 80–85% of embryogenesis are either swollen (Fig. 5D) or covered with knobby protrusions (Fig. 5C). These branches do not bear filopodia and would appear to have reached their final length, suggesting that this distinctive morphology results from some process other than growth – for instance, synaptogenesis with target cells. It seems unlikely

that these swollen or knobby branches are an artifact of the cobalt filling procedure because there is no distension or blebbing of the supporting axon shaft.

When the sensory axon becomes morphologically mature its branches lose their growth cones, filopodia, and knobby protrusions but often retain smooth 1–2 μm swellings at their tips. These structures are commonly seen on sensory axons in the adult grasshopper and are believed to be presynaptic boutons (Bacon & Altman, 1977). Mature-looking axons were first found at 80% of embryogenesis in the glomerular sensory shell, and exhibited branching patterns which were identical to afferents stained during postembryonic life. Presuming that the first sensory axons to differentiate in the periphery are also among the first to mature in the CNS, the complete morphogenesis of the sensory neuron occupies 25% of embryonic life (equal to 7–8 days at 30 °C.), and the formation of the central branching pattern encompasses 15% of embryogenesis (4–5 days) from the time the growth cone first enters the neuropil and contacts its target cells. All of the axons in the cercal afferent projection appear to be mature by 95%.

Embryonic formation of axonal branching patterns

By tracing particular axons or groups of axons at different stages in their growth it was possible to follow the sequence of events in branching pattern formation. The different types of sensory axons could be distinguished very soon after they enter the ganglion at 65% because they follow pathways which are characteristic of the adult sensory tracts. This means of identification would not be justified if the immature sensory axons send collaterals into more than one tract, but the earliest cobalt fills of sensory axons show individual axons projecting into a single sensory tract without sending branches or large numbers of filopodia into other available tracts (Fig. 6A). The branches which do sprout from these sensory tracts at later stages in embryogenesis also choose certain cell-type-specific pathways, and thereby establish the eventual domains of sensory axon arborization in their initial pattern of growth. The details of branching pattern morphogenesis are given below for each different axon type.

G Afferents. After following the cercal pioneer axons 5–10 μm into the terminal ganglion neuropil the G afferents split into three distinct groups which turn away from the pioneers. The first axons in each group choose virgin pathways which have not been taken by any preceding cercal afferent. The growth cones of later-arriving afferents follow along these first axon shafts (Fig. 6A). The different groups of G sensory axons encircle the future site of the glomerulus, and each group establishes one of the three sensory tracts seen in the mature glomerular shell (Fig. 6B). The tracts are packed with growth cones and surrounded by a halo of filopodia, but there are no branches projecting into the surrounding neuropil at this early stage.

Branches tipped with secondary growth cones were seen to be growing out of

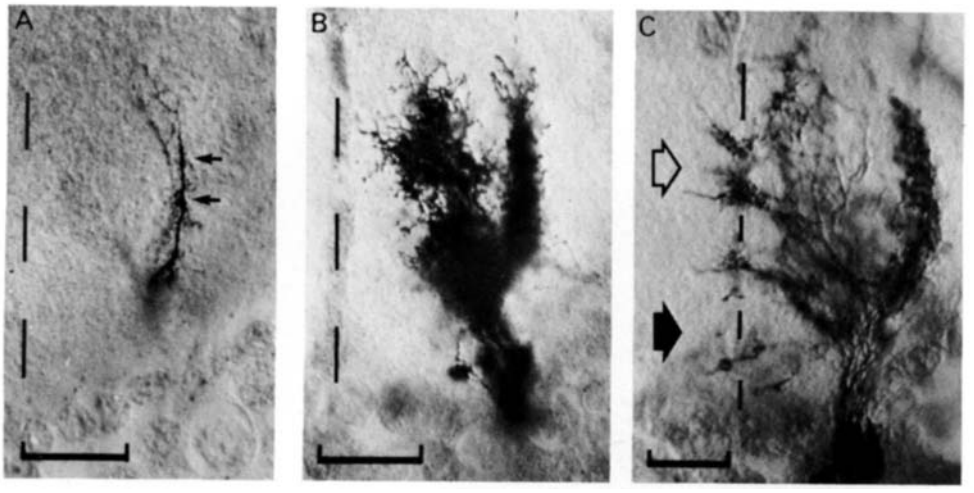
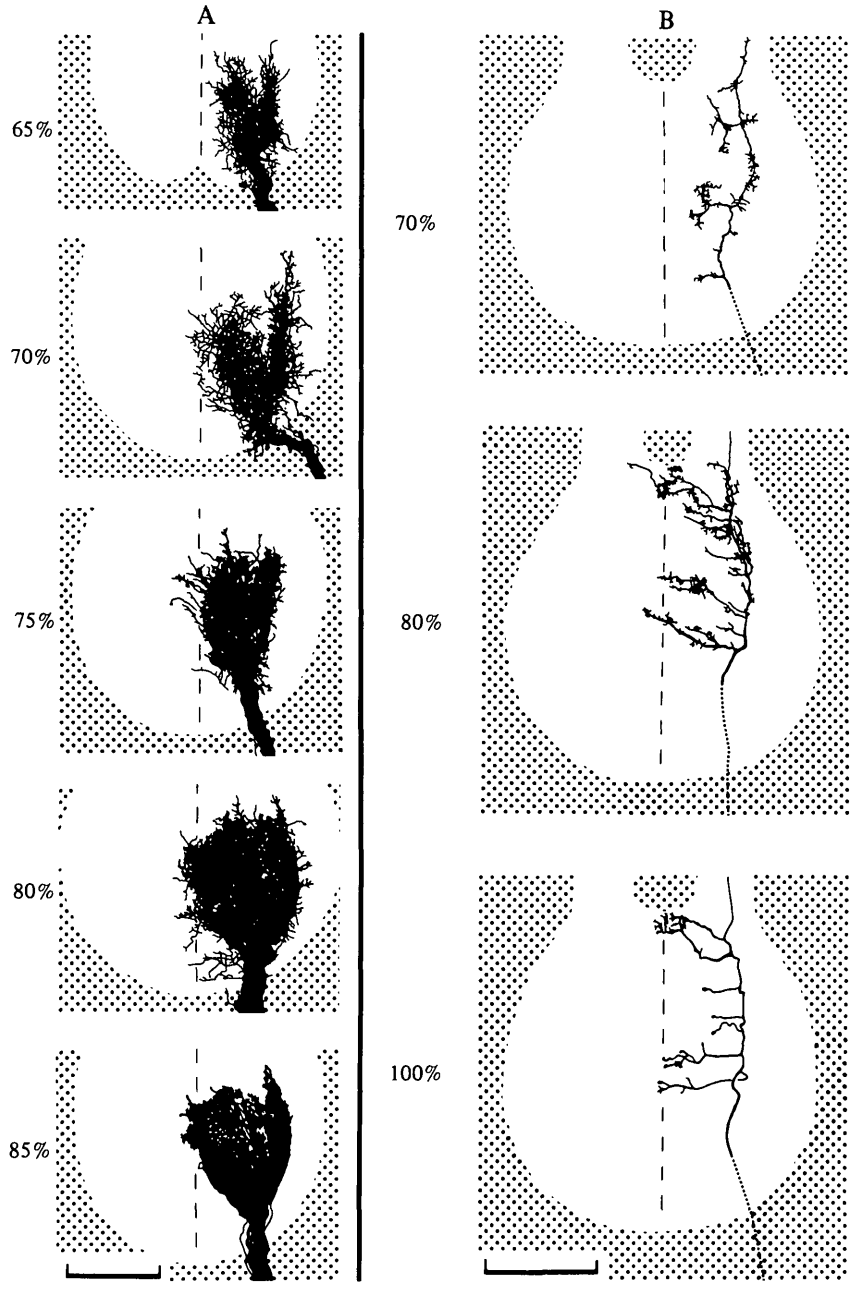


Fig. 6. Construction of the glomerular shell by embryonic sensory axons. Photomicrographs of 25 μm sections from silver-intensified cobalt fills. Midline marked by a broken line. (A) This fill shows a pair of axons which appear to be establishing the route of the lateral glomerular tract. Each axon terminates in a spindle-shaped growth cone (arrows) which has a terminal filament covered with filopodia extending from its tip. Note that the second growth cone is following along the shaft of the first axon, although there is a small separation in their paths near the edge of the neuropil. Comparison with part B shows that these axons do not have collaterals projecting into the future sites of the other sensory tracts (65% embryo). (B) As more sensory axons arrive in the CNS large numbers of growth cones come to occupy both the dorsal (left) and lateral (right) glomerular tracts. The filopodia emanating from these tracts do not reach the midline at this stage (65% embryo). (C) The glomerular sensory axons form a hollow shell of arborization by growing branches towards the midline. The growing tips of these branches (hollow arrow) establish a crossing projection in the posterior commissure of segment A_9. At this stage there are also transitory branches which extend toward the midline from the posterior portion of the glomerulus (solid arrow), but these processes soon disappear (80% embryo). Scales 20 μm.

the glomerular sensory tracts at 70–80%. The branches of the G_1 afferents take curved paths around the glomerulus, passing over the other two tracts and thus creating the continuous shell of sensory arborization characteristic of the adult. By comparing the outline of this shell at different embryonic stages, it can be seen that the G afferents arborize almost exclusively within the future boundaries of the glomerulus during the period of their initial growth (Fig. 7A). The filopodia arising from the growing sensory axons extend up to 20 μm outside these tracts, but very few actual branches project beyond the eventual edges of the glomerulus at any embryonic stage. The filopodia and inappropriate branches all disappear by 85%, leaving the perimeter of the glomerular shell of sensory arborization sharply defined.

L Afferents. Cobalt fills which were performed prior to 95% of embryogenesis stained only a single L sensory axon per cercal afferent projection. This axon first enters the neuropil at 65/70%, grows through the centre of the


A
B
65%
70%
75%
80%
85%
70%
80%
100%

developing glomerulus, and enters the lateral connective. Secondary growth cones then sprout from the sides of the L axon shaft and establish the cell's characteristic terminal ganglion branching pattern (Fig. 7B). These sidebranches first appear in the more posterior neuropil segments, which suggests that there is a fixed latency between the passage of the primary axonal growth cone and secondary growth cone formation.

The newly sprouted sidebranches of the L sensory axon point toward the midline without exception (Fig. 7B). This observation would seem to imply that the orientation of sidebranches in the mature branching pattern results from the initial pattern of growth, and is not dependent upon the pruning of branches which point in other directions. There is no evidence that any of the large, medially directed sidebranches are pruned either. Fills show that the growing L afferent, although covered with filopodia, has a branching pattern very similar to that seen in the hatchling and adult (Fig. 7B).

MV Afferents. The growth cones of the MN afferents begin to enter the terminal ganglion at 65% and pass through the neuropil along the cercal pioneer axon shafts, thus coming to occupy the same medioventrally located longitudinal tract. Sidebranches grow out of this tract at 65/70% and project throughout the ventral region of the ipsilateral neuropil.

A relatively small number of embryonic sensory axons have transitory branches which grow into regions of the neuropil that are not occupied in the hatchling or adult. In two different 65–70% cobalt fills there was an MV afferent branch (the longest one was 70 μm) whose growth cone had entered the contralateral side of the terminal ganglion neuropil posterior to the cercal glomerulus, where no such branches are seen at any other stage. There were also two cobalt fills performed at 70–80% which stained axonal branches extending toward the midline from the posterior region of the glomerular shell (Fig. 6C). However, branches with such markedly inappropriate projections were only seen in a few of the cobalt fills performed during embryonic life. Since each fill stains a very large number of axons (ranging up to approximately 100, depending upon the stage and the success of the filling procedure), the incidence of inappropriate projections was quite low at any given stage. This in turn suggests

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Fig. 7. Formation of sensory axon branching patterns. Camera-lucida tracings of silver-intensified cobalt fills. (A) While forming the sensory shell of the cercal glomerus the G axons arborize almost exclusively within their eventual domain. A large number of filopodia surround these tracts while the axons are still immature, but both filopodia and inappropriate branches recede by 85% of embryogenesis to leave the boundaries of the glomerulus sharply defined. (B) Morphogenesis of the L afferent's terminal ganglion branching pattern: 70%, the primary axonal growth cone has already passed through the terminal ganglion into the connective, and secondary growth cones have begun to sprout from the medial side of the axon shaft; 80%, the axon has established its basic branching pattern but is still covered with filopodia; 100%, filopodia and growth cones have disappeared and the branching pattern resembles that of the adult. Scales 50 μm.

that many of the cercal sensory axons form their embryonic branching patterns without growing outside the eventual domain of their arborization.

It is possible that some of the axonal branches which lie within the normal zones of sensory arborization also disappear as the axon grows, but this phenomenon could not be readily observed with the large numbers of axons stained by each fill.

Postembryonic development

After hatching from the egg, the grasshopper undergoes a series of 5–6 postembryonic moults before reaching sexual maturity and achieving its final size. There are no new central neurons generated in the terminal ganglion during this period of time (Gymer & Edwards, 1967), but the existing neurons grow larger and the volume of the terminal ganglion neuropil (measured from silver-stained sections and cobalt fills) increases approximately 15×. There are also postembryonic changes in the allometric relationships between different parts of the CNS – for instance, the cercal glomerulus occupies 20% of the hemi-ganglion neuropil volume at hatching, but only 10% in the adult.

There are no pronounced changes in the distribution of sensory axons within the CNS during postembryonic life (Fig. 1A). The major classes of cercal afferents are all represented in the embryonic projection, and no new sensory tracts or zones of arborization are added at any later stage. This means that the sensory axons which are present at hatching do not undergo any obvious branching pattern re-organization, and the sensory neurons which are generated in association with the postembryonic moults (comprising 75–85% of the sensory neurons in the adult cercus, Shankland, in preparation) send their axons through the neuropil along the pre-existing tracts without forming appreciably novel projections. If the consequences of postembryonic development are examined at the level of an individual branching pattern by comparing L sensory axons stained at hatching (Fig. 7B) and in the adult (Fig. 4A), it can be seen that there is no significant change in either the axon pathway or the number and location of the major branches. There is, however, a small increase in the degree of secondary branching of the L sensory axon during postembryonic life.

Anatomy and development of the MGI

The four largest axons in the grasshopper's abdominal nerve cord derive from the Giant Interneurons of the terminal abdominal ganglion (Cook, 1951). One of these axons is located medially, while the others form a cluster in the dorsolateral quadrant of the connective (Fig. 1C). The cell with the medially situated axon will henceforth be called the Medial Giant Interneuron (MGI). This name is in accordance with the nomenclature used for crickets (Edwards & Palka, 1974), but is not meant to necessarily imply that the grasshopper and cricket MGIs are phylogenetically related cells.

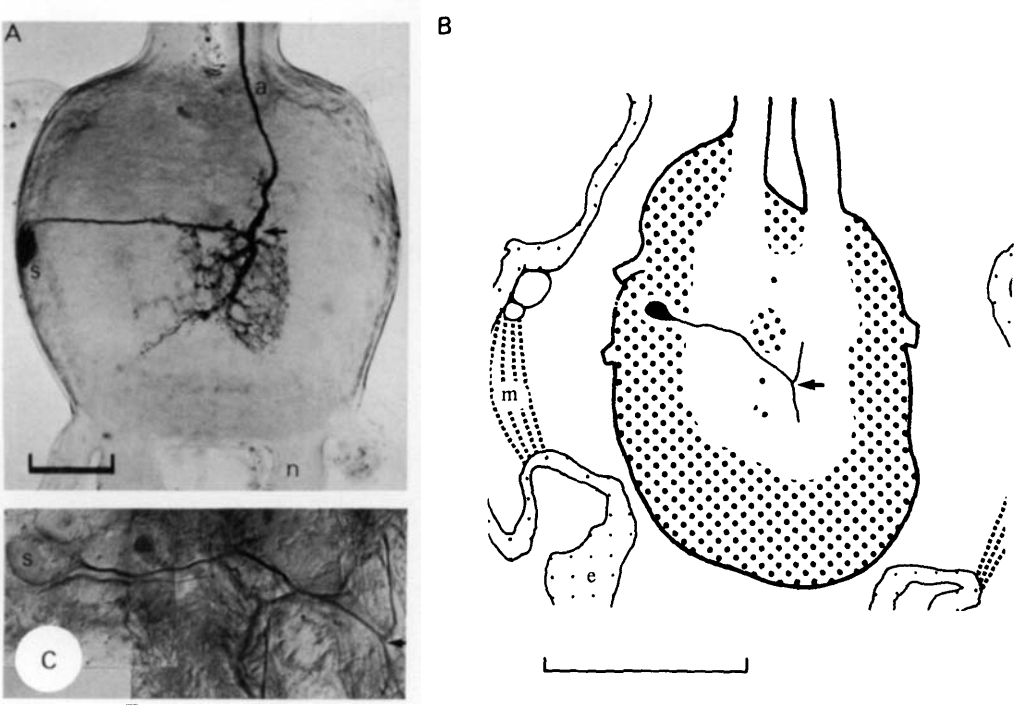

Fig. 8. Anatomy and embryonic appearance of the MGI. Arrow marks the T-junction of the neurite, the anteriorly directed axon (a), and the posteriorly directed dendrite. (A) Photomicrograph of a silver-intensified cobalt fill seen in a whole-mounted terminal ganglion. The main dendrite supports an egg-shaped cluster of secondary branches which occupy the interior of the cercal glomerulus. s, Soma; n, cercal nerve (Adult). Scale 100 μm. (B) The MGI traced from a silver-stained section of the terminal ganglion neuropil at the stage that the first sensory axons arrive in the CNS. The dendrite already occupies the eventual site of the glomerulus at this time. The ganglion lies adjacent to the body wall and is therefore bounded by epidermis (e) and muscles (m) (65% embryo). Scale 100 μm. (C) Composite photomicrograph of the specimen diagrammed in part B. This is the earliest stage that the MGI can be readily identified with the silver stain. The neurite of the left MGI can be traced from its soma (s) to the characteristic T-junction. The mirror image branching pattern on the MGI on the right side of the ganglion can also be seen (65% embryo).

The major process of the grasshopper MGI lie within the same horizontal plane as its cell body (Fig. 8). This soma is located dorsal and slightly posterior to the eighth ventral nerve near the equator of the ganglion. The neurite follows a straight path into the contralateral neuropil through the anterior commissure of segment A_9 and there forms a characteristic T-junction by dividing into an anteriorly-directed, interganglionic axon and a large, posteriorly-directed dendrite. The stout trunk of this contralateral dendrite projects into the centre of the cercal glomerulus and supports an egg-shaped cluster of finer branches which fill the interior of the glomerular sensory shell (Fig. 8A). A few of these branches cross the midline and arborize within the glomerulus that is ipsilateral to the soma.

Since the most distinctive parts of the MGI's anatomy can be captured in a single horizontal plane, this cell was easily recognized in sections of the embryonic neuropil which had been stained by the Rowell silver technique. Four criteria were used for identification: (i) soma location; (ii) neurite crossing through the anterior commissure of segment A_9; (iii) contralateral T-junction; and (iv) soma and processes appreciably larger in diameter than surrounding cells. In all embryos 65 % and older there were a bilateral pair of terminal ganglion neurons which fit this description and were therefore believed to be the MGI's (Fig. 8C). This means that at least the main trunk of the MGI's contralateral dendrite lies in the appropriate region of the terminal ganglion neuropil at the stage the first cercal sensory axons arrive. The MGI was not large enough to be readily distinguished from surrounding cells in silver stains performed at earlier stages, but preliminary intracellular dye injections have shown that the main trunk of this dendrite is actually present as early as 50 % (C. S. Goodman, M. Shankland & C. M. Bate, unpublished results).

DISCUSSION

The accompanying paper (Shankland, 1981) describes the peripheral pioneer axons which establish the nerve route leading from the grasshopper's cercus into its CNS during embryonic life. The present paper is an account of the growth and branching of the sensory axons which follow the pioneer nerve into the terminal abdominal ganglion at a later embryonic stage. The first of these sensory axons enter the ganglion at 65 % of embryogenesis, and grow through the neuropil for a short interval before forming branches. The morphology of the sensory axon is identical to that of the growing pioneer axon at this time. The axon has an ellipsoidal or spindle-shaped growth cone with a long terminal filament extending from its leading edge and short filopodia arising from the sides of both the filament and the axon shaft. The developing sensory axons then proceed to ramify through the neuropil by forming secondary growth cones which give rise to branches. These growth cones and filopodia recede prior to hatching, leaving the morphologically mature sensory axon terminals tipped in smooth swellings believed to be presynaptic boutons. Electrophysiological experiments have shown that the cercal sensory axons have formed functional central connexions by the time embryogenesis is complete (M. Shankland & D. Bentley, unpublished results).

The cercal sensory afferents were split into three general classes on the basis of their central projections: the glomerular (G) afferents, which have been subdivided into dorsal, ventral, and lateral tracts; the lateral (L) afferents; and the medioventral (MV) afferents. There is at least one afferent of each type in the embryonic complement of sensory axons, and the branching patterns seen at the time of hatching are the same as those found in the adult. The sensory neurons which send axons into the CNS during postembryonic life join these pre-existing classes without forming any additional sensory tracts.

However, this finding does not preclude the possibility that some of those sensory neurons which arise postembryonically have uniquely identifiable branching patterns because their individuality might be obscured in the whole nerve cobalt fills. For instance, the clavate hair sensory neurons of the cricket cercus constitute discrete classes, yet the individual axons within each class have distinctive branching patterns that vary systematically with respect to the cell's postembryonic birthdate (Murphey, Jacklet & Schuster, 1980). The failure of the grasshopper's postembryonic sensory afferents to form novel projections also implies that any developmental changes in the behavioural function of the cercus, such as its role in an exclusively adult behaviour like copulation (Gregory, 1965), do not arise from sensory axons invading previously unoccupied regions of the neuropil.

The development of the embryonic sensory axons was examined in more detail because they grow through the neuropil along virgin pathways and are therefore not obscured by preceding fibres. These axons choose one of the five basic sensory pathways of the adult projection (the MV, L, and three G afferent tracts) without exhibiting any noticeable period of diffuse or randomly directed growth. Furthermore, the cobalt-filled axons did not have collaterals extending into more than one of the available sensory tracts, which suggests that each type of sensory axon follows a specific pathway with a minimal amount of exploration of other alternative sensory axon routes (However, the present data cannot exclude the possibility that a sensory axon which encounters a choice point first grows along one path, then retracts to the choice point and grows along the second path. In this case there would be no collaterals, yet the axon would explore both paths.) The branches formed by each class of sensory afferent also grow within spatial constraints specific to that cell type. For instance, the branches arising from the immature L afferents grow exclusively toward the midline, while the G axons arborize almost entirely within the future boundaries of the glomerulus. Hence, the embryonic sensory axons achieve their mature central distribution with very little invasion of the remainder of the neuropil. This means that the mature branching patterns of the sensory axons are largely determined by their initial pattern of growth, and do not result from primary overgrowth followed by selective pruning of inappropriate branches.

Two kinds of limited overgrowth were observed during the development of the cercal afferent projection. The most prevalent form of overgrowth is the formation of filopodia by the axonal growth cone. These ephemeral processes extend up to 20 μm perpendicular to the axon shaft, and thereby offer the growing fibre direct contact with a cylindrical domain of neuropil surrounding the growth cone's path. Since filopodia accompany the growth of most neuronal processes (Johnston & Wessells, 1980), they represent the minimal degree of overgrowth one would expect to observe. The cercal sensory axons also produce actual branches, up to 70 μm in length, which grow into inappropriate regions

of the neuropil, then regress at a later stage. The formation of these transitory branches implies that the sensory axon has the capacity to selectively maintain only a specific subset of the processes that are established by its growth cones. The axon thereby refines the branching pattern laid down during its initial growth, and would appear to be eliminating branches whose growth cones have navigated inappropriate routes. However, the highly specific pattern of initial growth in the cercal sensory projection results in a small number of obviously inappropriate branches, so it is apparent that precise growth rather than selective maintenance is the principal determinant of sensory axon branching patterns. The relative importance of these two morphogenetic processes varies for other nerve cells. Some neurons, like the cercal afferents, have a quite specific pattern of initial growth (Lund, Booth & Lund, 1977; Bentley & Toroian-Raymond, 1981), while others normally rely upon selective maintenance to shape the final distribution of their branches (Rakic, 1977). It is also possible for a neuron to show a minimal amount of overgrowth in one region of the nervous system, such as the neuropil of the CNS, but produce many extensive transitory branches elsewhere (Goodman *et al.* 1979).

A precise, non-random pattern of initial growth indicates that the axon is being guided through the cellular landscape, although the nature and specificity of the guidance cues cannot necessarily be deduced. Such guidance cues are obviously important to the functional organization of the nervous system because an axon or other neuronal process must locate its target cell before it can form the appropriate connexion. Furthermore, it is possible that the set of guidance cues which are available to a growing axon could limit its access to potentially compatible partners, and thereby help to determine nervous system connectivity by avoiding contacts between compatible yet inappropriate pairs of cells. Physical access has proven to be crucial for synaptogenesis in experimental situations (Bixby & Van Essen, 1979), and may serve as a determinant for the pattern of synaptic connectivity during ontogeny as well. This sort of morphogenetic specificity has been recently gaining popularity as an alternative to 'chemospecificity' in explaining the patterning of synaptic connexions (Horder & Martin, 1978). Restrictions of cell contacts could only occur if the axon has a precise pattern of growth, such as that of the cercal sensory axons, which leaves large portions of the neuropil unexplored. For instance, the ingrowing cercal afferents do not invade the glomerulus on the contralateral side of the neuropil, although that region of the ganglion contains the dendrites of cells which are bilaterally homologous to their own synaptic partners. Transplantation experiments have shown that the sensory axons can synapse with these homologues (Palka & Schubiger, 1975), so the parameters which govern the pattern of growth of the sensory axon during normal development (e.g. available pathways; limited branching capacity) would seem to restrict its access to regions of the neuropil which contain obviously compatible partner cells.

The particular pathways chosen by the growing sensory axons permit speculation about the nature of the axonal guidance cues. In the periphery, the first sensory axons follow the nerve established by the pioneer axons, and it has therefore been proposed that the pioneers act as a source of guidance for the sensory axons en route to the CNS (Bate, 1976*b*; Edwards & Chen, 1979). Since one class of cercal sensory afferent, the MV axons, continue to follow the cercal pioneer axons within the neuropil, the pioneers might also be acting as guidance cues within the CNS. However, this correlation alone does not prove any causal relationship.

Synaptic partner cells are known to play an important role in the formation (Kimmel *et al.* 1977; Holland & Brown, 1980) and elimination (Goodman & Ridge, 1980) of neuronal processes, and may also be a source of guidance for axonal growth. In the cercal sensory system, interactions between the sensory axons and their central targets, the Giant Interneurons, may therefore be involved in shaping the precise and complementary branching patterns of these cells. This hypothesis is being tested by following the embryonic formation of the cercal glomerulus. In this paper it has been shown that the first embryonic G afferents turn away from the cercal pioneer axons to encircle the future site of the glomerulus, when they enter the neuropil, and that the main trunk of the MGI's dendrite is already present at that stage. Contacts with this dendrite may be serving to guide these axons away from the pioneer tract and into their eventual domains. Likewise, interactions with the sensory axons might be responsible for shaping the growth of the finer branches of the MGI.

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