J. exp. Biol. **112**, 45–64 (1984)
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FROM EMBRYONIC FASCICLES TO ADULT TRACTS: ORGANIZATION OF NEUROPILE FROM A DEVELOPMENTAL PERSPECTIVE

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SUMMARY

We discuss ideas emerging from our studies on selective axonal fasciculation in the grasshopper embryo that have implications for the organization of the adult neuropile in insects and perhaps other animals. While one of our laboratories has been studying the embryonic development of the G neurone (in the mesothoracic segment) and its lineal homologues (in other segments), the other has been studying the morphology and physiology of this same neurone and its segmental homologues in the adult nervous system. Our embryonic studies show that the growth cone of the G neurone selectively fasciculates with the A/P fascicle in preference to all other longitudinal axon fascicles as it turns anteriorly. The homologues of G in other thoracic and abdominal segments fasciculate in this same bundle. However, early in their morphological differentiation, they reveal interesting segmental differences. Our studies on the adult nervous system show that the segmental homologues of the G neurone share many properties in common (e.g. axons in the LDT: lateral dorsal tract) while other features are quite different. The notion emerging from these studies is that a basic segmentally-repeated pattern arises during embryogenesis: a stereotyped axonal scaffold upon which growth cones faithfully fasciculate. Evolutionary plasticity allows the specialization of lineally equivalent neurones in different segments within the context of the neuropilar neighbourhood that they find themselves in as a consequence of their selective fasciculation.

INTRODUCTION

During the 19th century, many of the first descriptions of invariant 'identified' neurones focused on giant axons which were repeatedly located in particular regions of the nerve cord. This observation of stereotyped axon location was best exemplified two decades ago by Wiersma and his colleagues who stripped away, recorded from, and stimulated bundles, or individual axons, in specific portions of the crayfish ventral nerve cord (e.g. Wiersma, 1958; Wiersma & Hughes, 1961; Wiersma & Ikeda, 1964). They extended the notion of stereotyped axon location beyond giant fibres and

Key words: Embryogenesis, neuroembryology, neuropile.

showed that the many different identified axons were located in specific regions of the connective.

But what difference does this make to neuronal specificity, since few synapses occur in the connective? The stereotyped axon locations in the connectives are likely to reflect the more complex pattern of axon associations in the ganglionic neuropile where most synapses occur. With the advent of intracellular dye injection techniques (e.g. Stretton & Kravitz, 1968; Pitman, Tweedle & Cohen, 1972; Stewart, 1978), many neurones became individually identified according to the location of their axons and dendrites in the neuropile. Although at first neurone morphology was described on a featureless background, some studies began to focus on the relationship of identified axons to other processes in the neuropile (e.g. Stretton & Kravitz, 1973).

In an attempt to relate the location of axons to one another within the neuropile, Tyrer & Gregory (1982) described nine major longitudinal axon tracts in each hemisegment, and the commissural tracts connecting the two hemisegments, in the neuropile of the grasshopper thoracic ganglia. They showed that the axons of some identified neurones run in specific tracts within this framework.

Given the specificity of neuronal connectivity, we wondered if there were further subdivisions of axonal associations within these nine major longitudinal tracts. Furthermore, we wondered how this axonal framework is constructed during development. Do dendrites faithfully fasciculate within stereotyped tracts as do axons? How do individual axons and dendrites find their way into these tracts? Just how specific is the association of certain axons and dendrites with one another? And finally, if each segmental ganglion is built on the same axonal framework, how can we account for segmental differences?

These and other questions concerning the functional organization of the arthropod neuropile might best be answered from the perspective of the embryo, where the tangled neuropilar fabric is less densely woven. Fortunately, the grasshopper embryo is ideal for such studies because the individual neurones are large and accessible, and the individual fascicles in which their axons run are spatially distinct and arranged in a simple orthogonal array. Previous studies on the grasshopper embryo led to the principle of growth cone guidance by selective fasciculation (e.g. Goodman, Raper, Ho & Chang, 1982; Raper, Bastiani & Goodman, 1983*c*, 1984; Bastiani, Raper & Goodman, 1984; Goodman, Bastiani, Raper & Thomas, 1984). Here we show how this model helps explain some of the organizational features of the adult neuropile. Furthermore, this model helps simplify the problem of neuronal specificity by reducing much of it to a series of cell recognition choices based largely on the affinities displayed by growth cones and their filopodia for particular axon surfaces.

Fig. 1. The G neurone in the mesothoracic (T2) and metathoracic (T3) ganglia of the adult grasshopper. *Camera lucida* drawings of Lucifer Yellow filled neurones. G(T3), previously named the B1 neurone, is lineally equivalent to G(T2) (i.e. arises from the 2nd division of NB 7-4). The contralateral primary axon of both cells extends anteriorly in the lateral dorsal tract (LDT) while the symmetrical dendrites extend anteriorly in the ventral intermediate tracts (VIT). Notice that G(T3) has a large secondary dendrite on the ipsilateral side that is missing in G(T2), while G(T2) has a contralateral secondary axon that is missing in G(T3). In addition G(T2) has symmetrical dendrites in contrast to the striking asymmetry exhibited by G(T3).

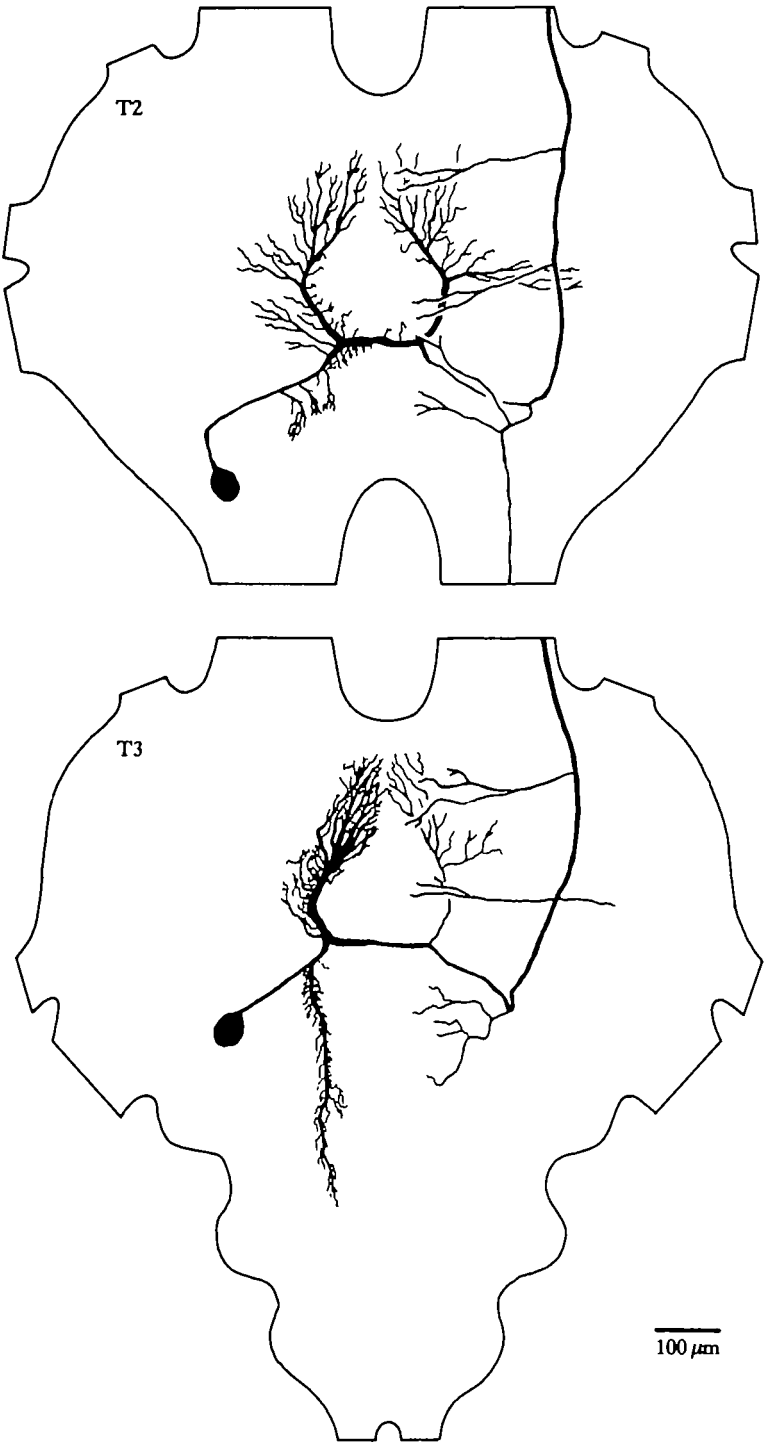


Fig. 1

THE G NEURONE, ITS SIBLING AND ITS HOMOLOGUES

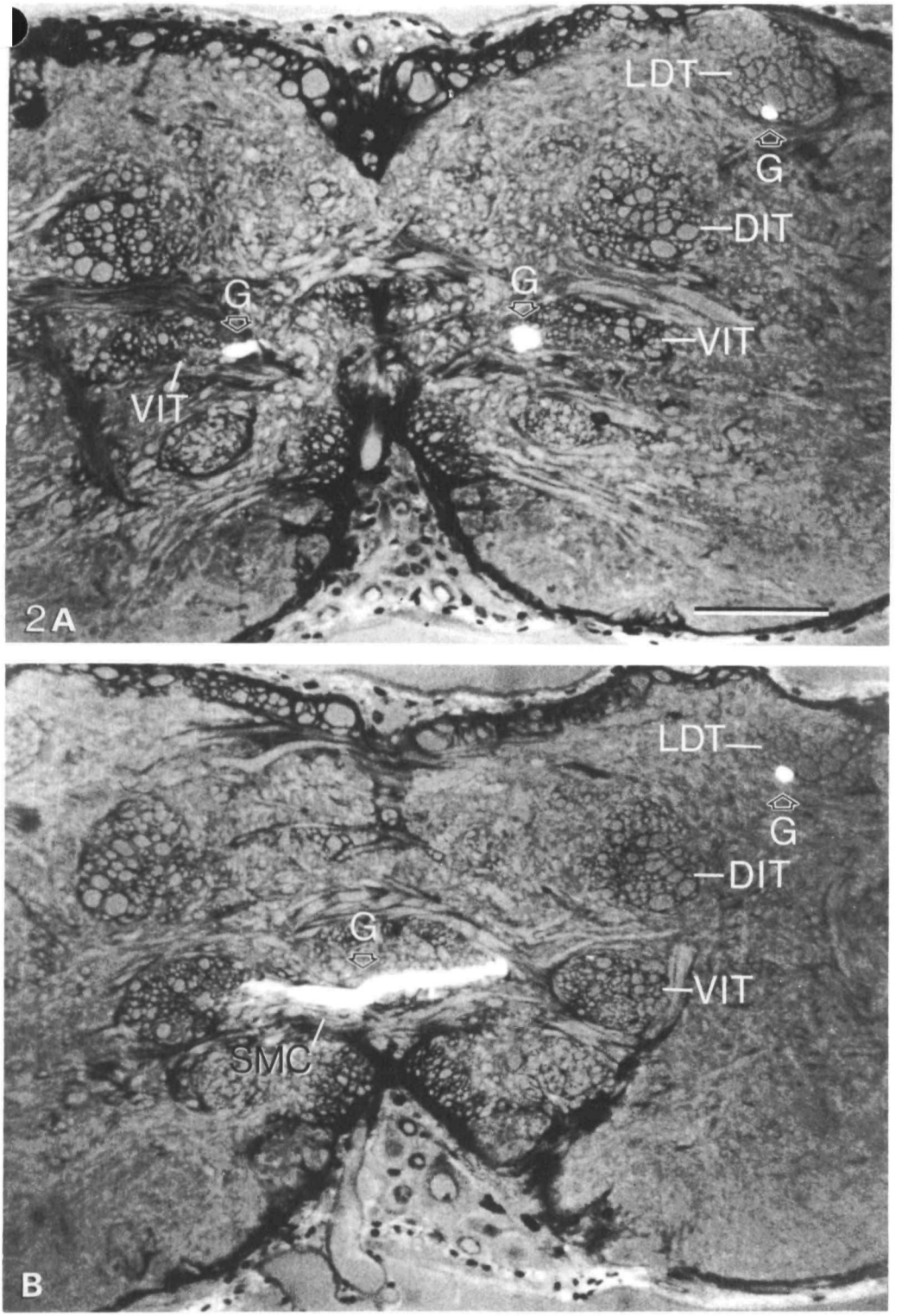
The studies we describe here are focused primarily on the G neurone in the mesothoracic (T2) ganglion of the grasshopper, as shown in Fig. 1 (Rehbein, 1976; Pearson, Heitler & Steeves, 1980). The T2 ganglion has about 2000 neurones, largely arranged as 1000 pairs of symmetrical bilateral homologues. Although many of these 1000 neurones can be identified, the G neurone is particularly well suited for our studies because it is highly accessible in both the embryo and adult, and much is already known about its development, morphology and physiology.

We penetrated the G neurone in the neuropile of the T2 ganglion, filled it with Lucifer Yellow, and then fixed and processed the ganglion for 10 μm plastic serial sections. The G neurone's cell body is situated posteriorly on the dorsal surface of the ganglion. Its neurite extends across the neuropile just dorsal to the supramedian commissure (SMC, see Tyrer & Gregory, 1982); its major axon then extends anteriorly in the contralateral lateral dorsal tract (LDT); its smaller axon extends posteriorly in a different contralateral tract, and its two primary dendrites extend anteriorly along the medial edge of both the ipsilateral and contralateral ventral intermediate tract (VIT) (Fig. 2). These tracts appear to establish both the axon's and dendrite's neighbourhood. Thus, we would predict that the G dendrites receive their inputs from axons in one neuropilar region while its two axons have their synaptic outputs in very different regions. This interpretation is supported by the finding that the axons of auditory afferents, one of the major monosynaptic inputs to G(T2), run in the same tract as does G's primary dendrite, the VIT (Tyrer & Gregory, 1982).

The G neurone and its immediate sibling, the C neurone (Pearson & Robertson, 1981), arise during embryogenesis from the 2nd division of neuroblast NB 7-4 (Raper *et al.* 1983*a*). Although the G and C neurones are siblings, they nevertheless differentiate into distinctly different neurones based on the opposite polarities of their primary axons, the locations of their primary dendrites in different longitudinal tracts, the different locations of their secondary axonal branches, and the subsequent differences in their synaptic inputs and outputs. The differences between the G and C neurones are first detected at about 40% of development when their primary growth cones make divergent fasciculation choices when confronted with the same embryonic environment (Raper *et al.* 1983*b*).

An example of an identified neurone in the metathoracic (T3) ganglion, called B1, is shown in Fig. 1 (Rehbein, 1976). Although B1's asymmetrical dendrites, lateral axonal branches and ipsilateral posterior dendrite appear quite different from G(T2), it also bears some striking similarities in terms of its general shape, and the location of its primary axon in the LDT and dendrites in the VIT. Embryonic cell lineage analysis reveals that the B1 neurone is the metathoracic homologue of G(T2)

Fig. 2. The tracts containing the primary axon and two primary dendrites of G(T2). Two thick plastic cross sections through the mesothoracic ganglion of an adult grasshopper in which G(T2) had been filled with Lucifer Yellow. (A) The G neurone extends its primary axon anteriorly in the contralateral lateral dorsal tract (LDT) and its symmetrical dendrites bilaterally in the ventral intermediate tracts (VIT). (B) The G neurone crosses to the contralateral neuropile just dorsal to the supramedian commissure (SMC) before extending its dendrite in the (VIT) and primary axon in the (LDT). (DIT) is the dorsal intermediate tract. Scale bar is 100 μm.


LDT
G
DIT
G
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2A
LDT
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DIT
G
VIT
SMC
B

i.e. lineally equivalent), and thus we refer to it as G(T3). Rehbein (1976) described another interneurone, called B2, whose cell body we find to be in the first abdominal (A1) segment. Cell lineage analysis again reveals that the B2 neurone is the first abdominal homologue of G and thus we refer to it as G(A1). The morphology of G(A1) and two additional G homologues, G(T1) and G(A2), are described later in this paper.

EMBRYONIC NEURONES, PRECURSORS AND LINEAGES

Embryogenesis in the grasshopper takes 20 days at 33 °C; each day equals 5 % of development. The grasshopper's CNS is segmentally arranged with a chain of cephalic, thoracic (~2000 neurones each) and abdominal (~500 neurones each) ganglia. Individual identified neurones are large and accessible from their birth to maturity, and the cell bodies of many can be recognized with Nomarski optics simply on the basis of their characteristic location and size.

The largely bilaterally symmetrical identified neurones in each segmental ganglion are generated by a precise pattern of precursor cells: two bilaterally symmetrical plates of 30 neuroblasts (NBs) arranged in seven rows (Bate, 1976), an unpaired median NB

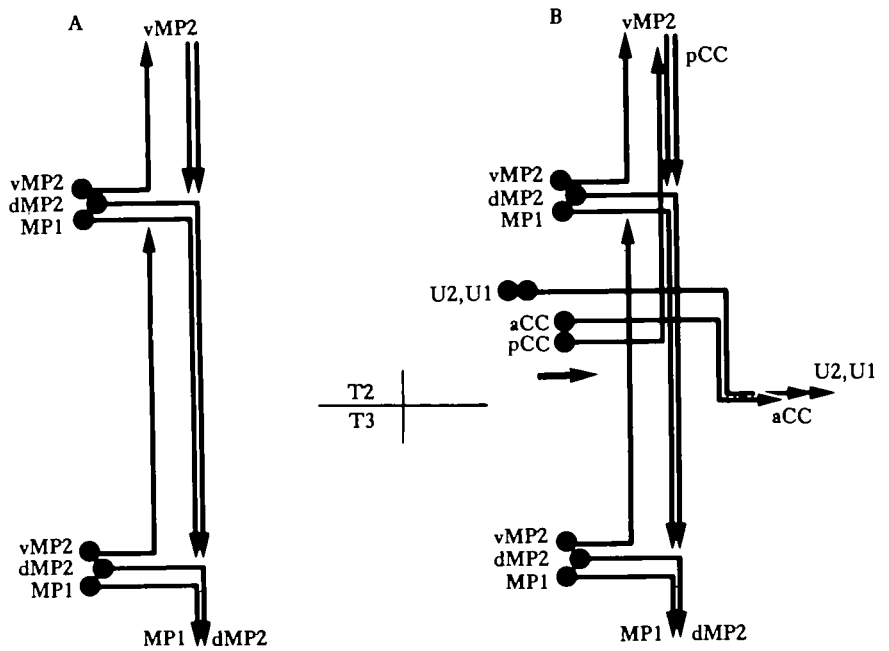


Fig. 3. Schematic diagram of the first three longitudinal axon pathways in the grasshopper embryo and the seven identified neurones whose axons fasciculate to pioneer these three fascicles: MP1, dMP2 and vMP2 (siblings), aCC and pCC (siblings), U1 and U2 (siblings). (A) The pioneering of the first two longitudinal pathways by the MP1, dMP2 and vMP2 growth cones. Note that MP1 and dMP2 fasciculate as they extend posteriorly, whereas the vMP2 extends anteriorly and pioneers a more medial (and ventral) pathway; the vMP2 does not fasciculate with the MP1 and dMP2 growth cones when they meet near the segment border. (B) The third longitudinal fascicle is pioneered by the U1 and U2 growth cones. The pCC growth cone extends anteriorly along the MP1/dMP2 fascicle; the aCC growth cone extends posteriorly along the U fascicle. The large arrow in B marks the approximate location of the electron micrograph shown in Fig. 4B.

(MNB), and seven midline precursors (MPs) called MP1, $MP2_L$, $MP2_R$, MP3, MP4, MP5 and MP6 (Bate & Grunewald, 1981). Each NB is a stem cell, maintaining its large size as it divides repeatedly to produce a chain of smaller ganglion mother cells (GMCs). Each GMC in the chain divides once more, thus producing a chain of paired ganglion cells which subsequently differentiate into neurones.

NBs each generate from 6 to 100 neuronal progeny, depending upon the NB, and then die. NBs generate neuronal progeny by invariant cell lineages; particular identified neurones arise from specific ganglion mother cells in the NB family trees (e.g. Goodman & Bate, 1981; Raper *et al.* 1983*a*; Taghert & Goodman, 1984). For example, the 2nd division of NB 7-4 generates a GMC which divides to give rise to the G neurone and the C neurone (Raper *et al.* 1983*a*). The 1st division of NB 1-1 generates a GMC which divides to give rise to the anterior (aCC) and posterior (pCC) corner cells (Goodman *et al.* 1982).

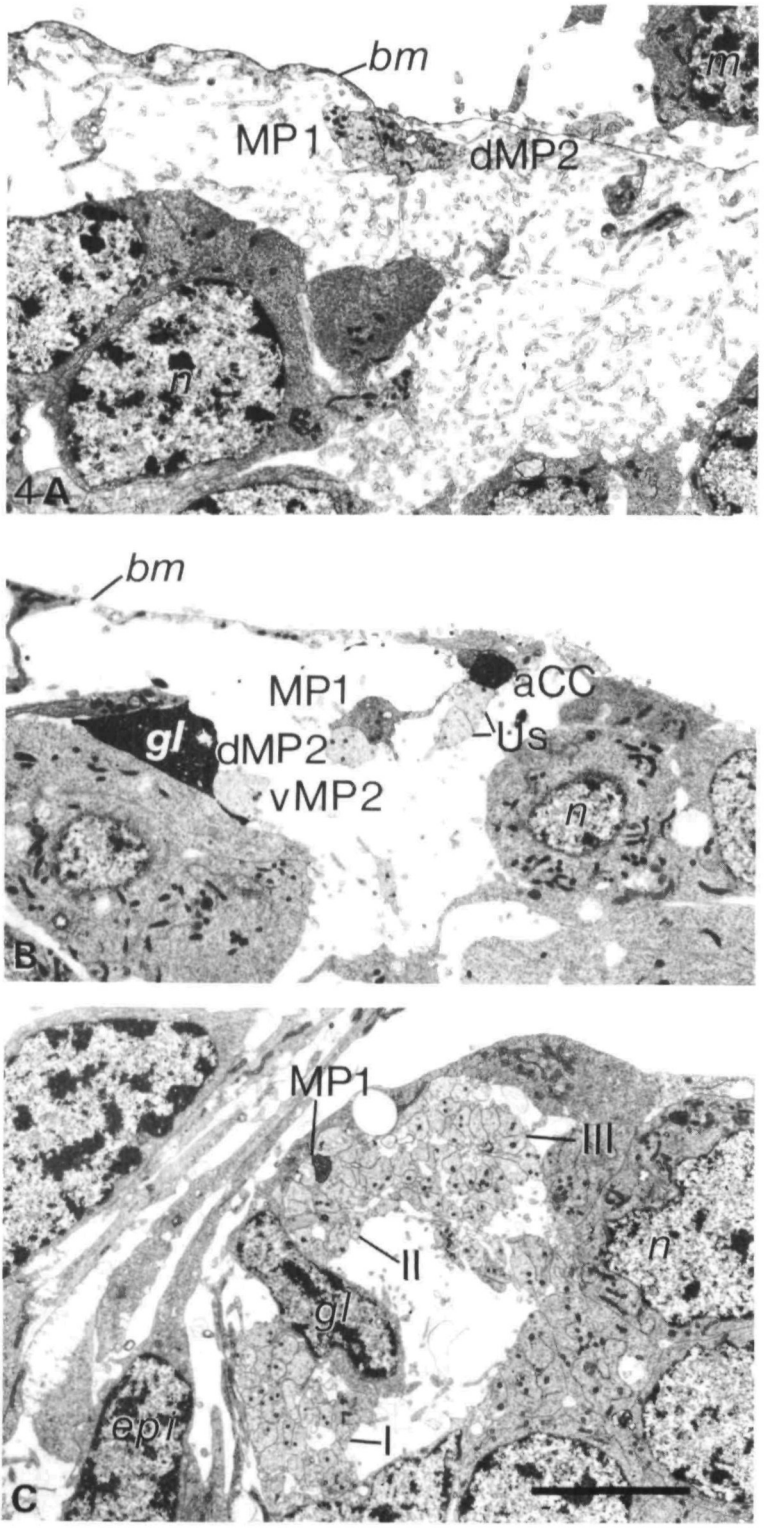
MPs divide only once to produce two neuronal progeny. In some cases, the two MP progeny behave quite similarly. In other cases, as for example with MP2, the two progeny behave quite differently, as described below.

DEVELOPMENT OF FIRST LONGITUDINAL AXON FASCICLES

The first two longitudinal axonal pathways in the CNS are pioneered by the growth cones of the MP1, dMP2 and vMP2 neurones (Bate & Grunewald, 1981). Each MP2 (one on each side) divides once to give rise to a ventral (vMP2) and dorsal (dMP2) daughter cell. The single MP1 gives rise to a pair of bilaterally symmetrical daughters, each of which comes to lie dorsal to the two MP2 progeny, thus forming a trio of cells on each side. All three cells (on each side) send growth cones up to the dorsal basement membrane at about 29% of development. After several hours, and irrespective of their initial orientations relative to one another, the growth cone of the vMP2 extends anteriorly, while the growth cones of the dMP2 (its sibling) and the MP1 extend posteriorly (Fig. 3A).

The MP1 and dMP2 growth cones extend posteriorly along the dorsal basement membrane (Fig. 4A) and use the pCC neurone as one of their specific guidance cues (Goodman *et al.* 1982; Taghert, Bastiani, Ho & Goodman, 1982; Bastiani & Goodman, 1984*a*,*b*; M. Bastiani, unpublished). The vMP2 growth cone extends anteriorly

Fig. 4. The development of the first three longitudinal axon fascicles and the subsequent three superfascicles in the ventral nerve cord of the grasshopper embryo. (A) The MP1 and dMP2 neurones send the first growth cones posteriorly along the dorsal basement membrane (*bm*) at 31% of development. Note the space under the basement membrane densely filled with filopodia, especially when compared to the spaces in B and C. (B) The first three longitudinal fascicles (the vMP2 fascicle, the MP1/dMP2 fascicle and the U fascicle) at 35% of development contain the vMP2, MP1 and dMP2, and the U1, U2 and aCC axons, respectively. The pCC axon extends anteriorly along the MP1/dMP2 fascicle and is not present in this plane of section. The vMP2 growth cone extends anteriorly in a more ventral and medial position along a glial cell (*gl*) at the same time as the MP1 and dMP2 growth cones extend posteriorly in a more dorsal and lateral position. The Us (U1 and U2) and aCC take an even more lateral and dorsal position. In this preparation, MP1, the aCC and the glial cell have been filled with horseradish peroxidase. (C) At 42% of development many axons have joined the first three fascicles in the connective to form three major 'superfascicles': I, II and III. The MP1 neurone was filled with HRP to confirm its identity (and the MP1/dMP2 fascicle) within superfascicle II. *n*, neurones; *epi*, epidermal cells; *m*, mesodermal cell. Scale bar, 10 μm.


bm
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MP1
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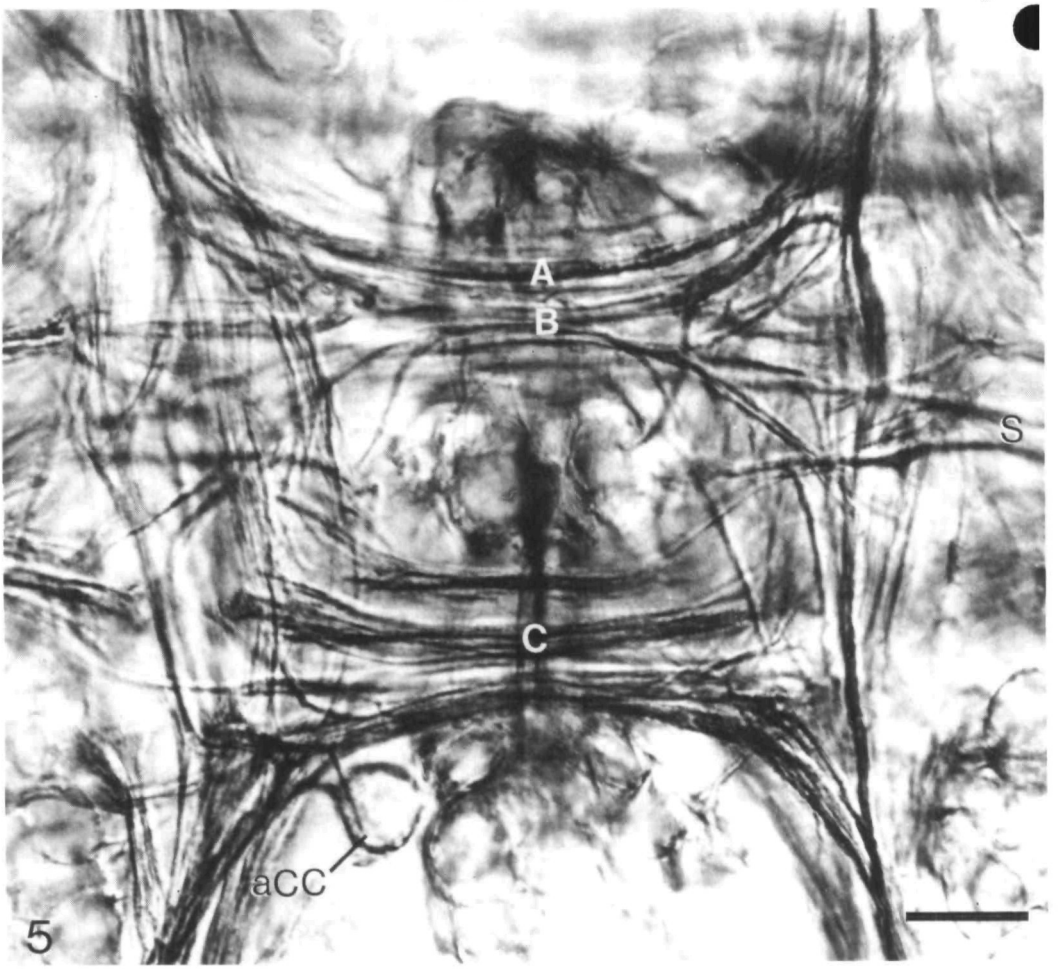


Fig. 5. The embryonic axon scaffold: an orthogonal array of axon fascicles in the neuropile of the T2 segment in a 42 % developed embryo. Dorsal view with Nomarski optics of a single focal plane of the whole-mount neuroepithelium stained with the 1-5 monoclonal antibody and a horseradish peroxidase-conjugated second antibody. A, B and C: the two anterior and one posterior commissure, respectively. S: the segmental nerve at the segment midline that will form nerves 3, 4 and 5 in the adult (IS, the intersegmental nerve at the segment boundary, is not shown). aCC: anterior corner cell. Anterior is to the top; scale bar, 25 μm.

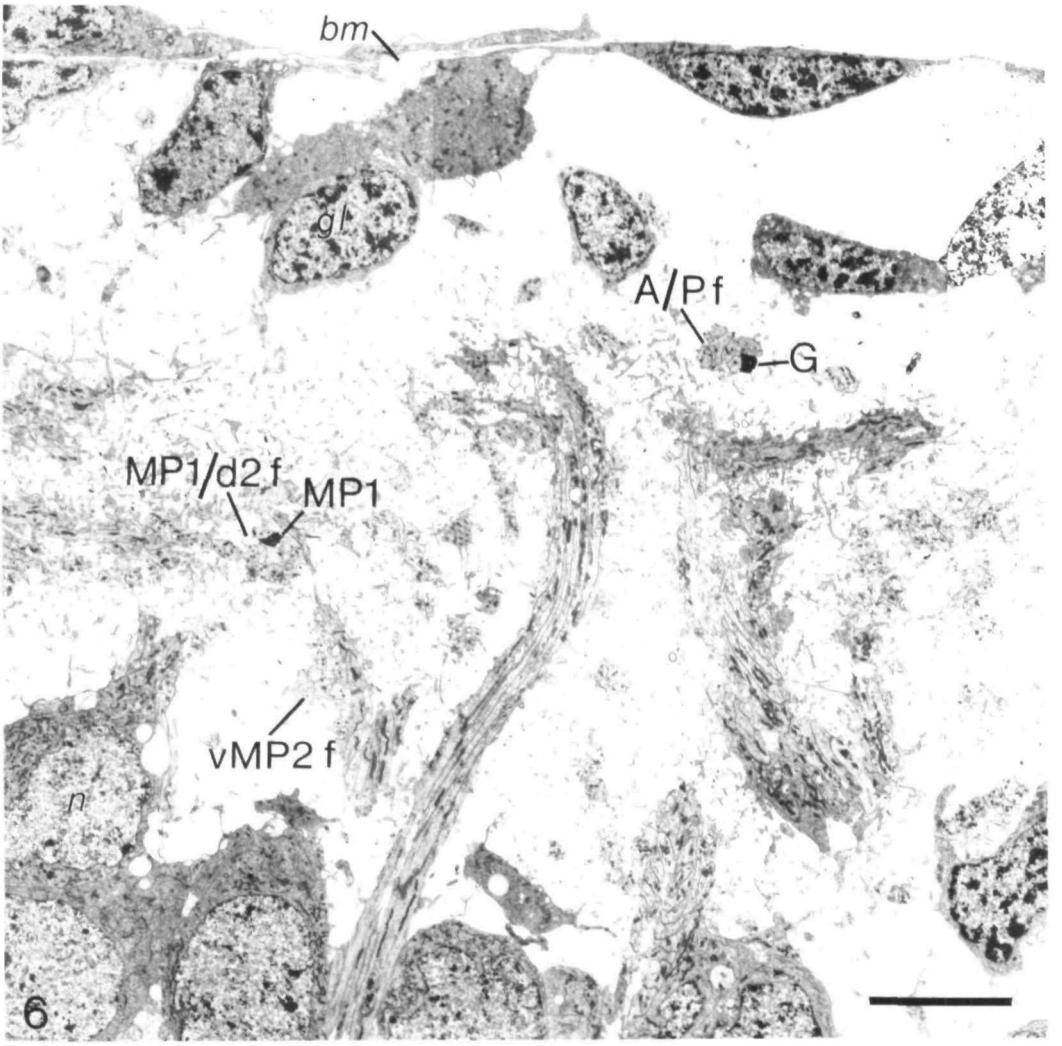


Fig. 6. Electron micrograph montage of the embryonic neuropile of a 42 % developed T2 ganglion. Cross sectional view taken at the level of the C commissure showing the axon fascicles in the right neuropile. This section is at the level of C in Fig. 5 and at the same age. There are about 25 longitudinal axon fascicles containing about 100 axons. The MP1 neurone within the MP1/dMP2 fascicle (MP1/d2 f) and the G neurone within the A/P fascicle (A/P f) have been filled with HRP. MP1 and dMP2 pioneer one of the first longitudinal axonal fascicles (MP1,d2 f) and vMP2 pioneers another (vMP2 f). The fascicles to the left (towards the midline) of MP1 d2 fascicle represent commissural fascicles interconnecting the two hemisegments. *bm*, basement membrane; *n*, neurone; *gl*, glia. Scale bar, 25 μm.

and appears to use other specific neuronal landmark cells as guidance cues. When the vMP2 growth cone meets the dMP2 and MP1 growth cones from the next anterior segment, they do not fasciculate; rather, the MP1 and dMP2 extend posteriorly along the dorsal basement membrane whereas the vMP2 extends anteriorly along the ventromedial glial cell (Fig. 4B). Thus, the first two longitudinal fascicles are pioneered by the MP1 and MP2 progeny (Fig. 3A).

The pCC growth cone extends anteriorly and fasciculates with the posteriorly extending MP1 and dMP2 growth cones (Fig. 3B). The aCC growth cone, on the other hand, appears uninterested in either of these two fascicles. Rather, it waits another 12 h until about 35 % of development when the U1 and U2 axons extend posteriorly just lateral to the MP1/dMP2 fascicle, thus pioneering the third longitudinal fascicle (Figs 3B, 4B). The aCC growth cone then extends posteriorly, fasciculating with the U axons (Bastiani, DuLac & Goodman, 1984; S. DuLac & C. S. Goodman, in preparation). In this way, the specific patterns of selective fasciculation by these seven axons establish the first three longitudinal axon fascicles by about 35 % of development (Fig. 4B shows only six axons because the pCC from the next posterior segment is extending anteriorly along the MP1/dMP2 fascicle and has not yet reached the location of this section).

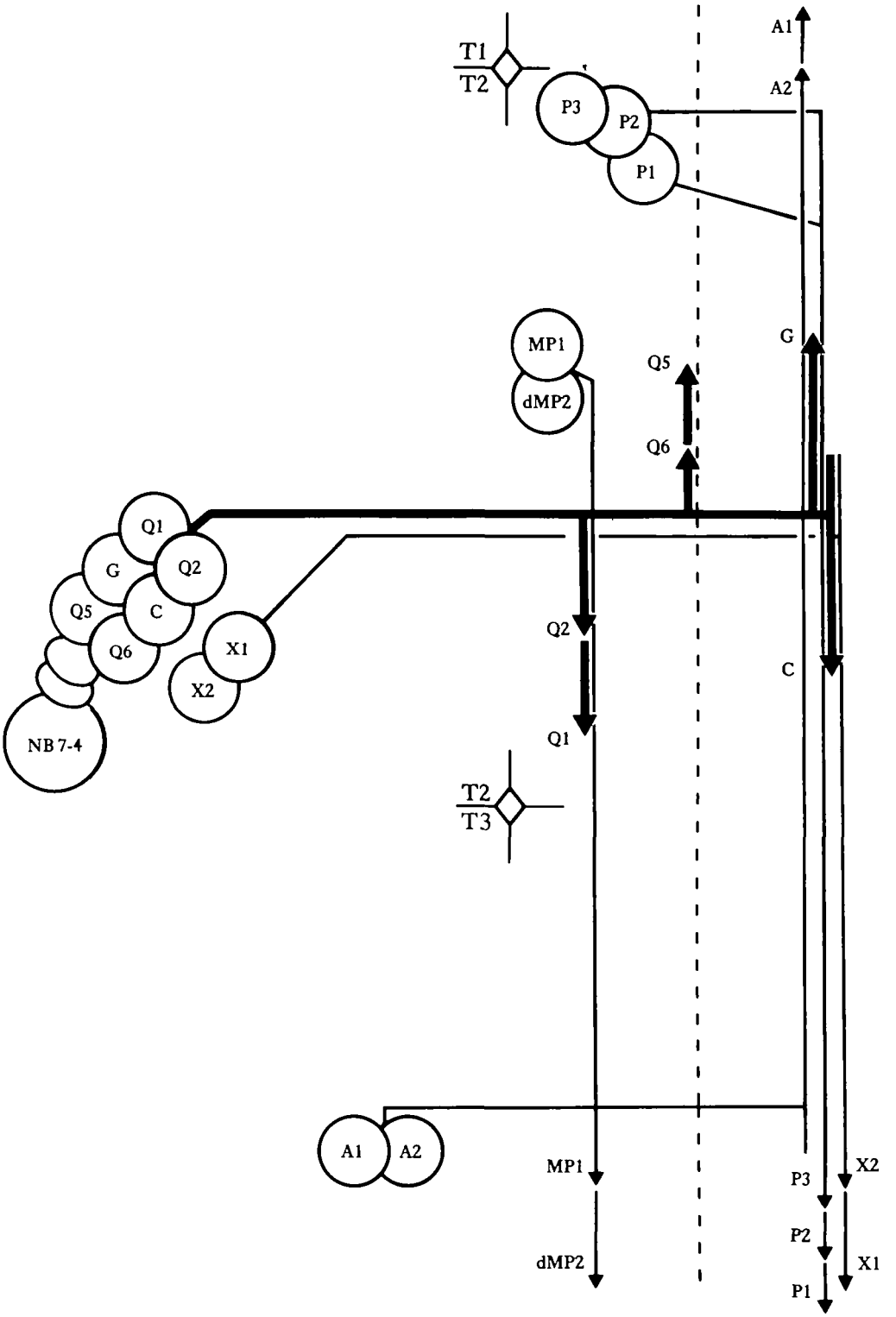
DEVELOPMENT OF THE NEUROPILE

Many additional longitudinal axon fascicles continue to develop in the neuropilar space between 35 % and 42 % of development. Viewed through the light microscope from its dorsal surface, the neuropile of a single segment appears as an orthogonal scaffold of axon fascicles (Fig. 5). Viewed in the electron microscope from cross section, each hemisegment contains about 25 longitudinal axon fascicles (containing about 100 axons) (Fig. 6). In addition to these longitudinal fascicles, each repeated segmental unit of the scaffold contains three commissures each with about 20 fascicles, and many other fascicles extending peripherally in the intersegmental nerve (IS) at the segment boundary (forming adult nerves 1 and 6) or the segmental nerve (S) at the segment midline (forming adult nerves 3, 4 and 5) (Bate, 1983).

Each of these fascicles appears to be highly invariant in its location and its membership from side to side, segment to segment, and embryo to embryo. It is upon these axon fascicles that later growth cones make their decisions about which way to grow, and it is within the neighbourhoods provided by these fascicles that they subsequently decide with which cells to synapse.

DIVERGENT CHOICES BY GROWTH CONES OF RELATED NEURONES

The first six progeny of NB 7-4 are called Q1 and Q2, G and C, and Q5 and Q6 (Fig. 7). Q1's growth cone meets the growth cone of its contralateral homologue Q1 at the midline, and there they fasciculate on one another to form one of the first of the eventual ~20 axon fascicles in the posterior commissure (Goodman *et al.* 1982; Raper *et al.* 1983*a*). Although several different axon fascicles develop shortly thereafter in the posterior commissure, Q1's next five siblings fasciculate upon the Q1 fascicle.


T1
T2
P3
P2
P1
A1
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G
MP1
dMP2
Q5
Q6
Q1
G
Q2
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C
Q6
X1
X2
NB7-4
Q2
Q1
C
T2
T3
A1
A2
MP1
dMP2
P3
X2
P2
X1
P1

Fig. 7

However, upon reaching the contralateral side of the developing neuropile, the growth cones of these clonally related neurones diverge as they make cell-specific choices as to which way to grow (Raper *et al.* 1983*a*). As shown schematically in Fig. 7, the growth cones of Q1 and Q2 turn posteriorly along the MP1/dMP2 fascicle, G and C turn in opposite directions along the more lateral A/P fascicle (containing the A and P axons as described below), and Q5 and Q6 turn anteriorly along a different intermediate pathway (Raper *et al.* 1983*b*). Thus, the primary growth cones of each of these neurones diverge in the neuropile as each selectively fasciculates with a particular axon bundle.

DIVERGENT CHOICES BY GROWTH CONES OF THE SAME NEURONE

At 40 % of development, the primary growth cone of the G neurone turns anteriorly in the dorsolateral A/P fascicle (Fig. 8B). By 45 %, it has already reached the next anterior segment, and continues to extend anteriorly in the A/P fascicle to reach the brain. By 50 % of development, a secondary axonal growth cone has begun to extend posteriorly in a more medial axon fascicle. At about the same time, the two primary dendritic growth cones extend anteriorly in bilateral locations, fasciculating with the MP1/dMP2 bundle (Fig. 2).

Each of these subsequent G growth cones diverges from the primary axon in the neuropile as they selectively fasciculate with different axon bundles. Presumably, later secondary dendritic and axonal branches make yet further divergent choices by their selective fasciculation with other processes. Taken as a whole, the range of divergent choices made over time by all of the G growth cones is equivalent in many respects to the same range of choices shown by the primary growth cones of the first six NB 7-4 progeny. The ability of subsequent dendritic and axonal growth cones to make fasciculation choices that are different from the primary axonal growth cone suggests temporal and/or spatial changes in the surface molecules on the G neurone that dictate its affinity for other neuronal surfaces.

DEVELOPMENT OF A/P FASCICLE

As the primary growth cone of the G neurone turns anteriorly, it fasciculates upon a discrete bundle of axons in preference to other nearby bundles (Fig. 8C). This bundle, called the A/P fascicle, at the stage that G initially fasciculates with it, contains the axons of the A1, A2, P1 and P2 neurones (Fig. 8B). The P1 growth cone pioneers the A/P fascicle as it extends posteriorly along the dorsal basement membrane (Fig. 8A). It is followed by the P2 axon. The posteriorly growing axons of the

Fig. 7. Schematic diagram of the divergent choices made by the growth cones of the first six progeny of NB 7-4. All six growth cones choose the same pathway across the posterior commissure, the Q1 fascicle, yet make divergent choices in the contralateral neuropile. Q1 and Q2 turn posteriorly upon the MP1/dMP2 fascicle. G extends anteriorly upon the A/P fascicle. C extends posteriorly in this same axon bundle once other axons (including X1 and X2) have joined the bundle. Q5 and Q6 extend anteriorly in a different, more medial pathway (dashed line). T1/T2 and T2/T3 represent the segmental boundaries. Notice that the Ps are from the same segment as G and C, while the As are from the next posterior segment.

P1 and P2 neurones meet and fasciculate with the two anteriorly growing axons of the A1 and A2 neurones. The axon of the P3 neurone joins the fascicle shortly thereafter. The A/P fascicle becomes displaced about 20 μm from the basement membrane; this membrane subsequently becomes covered by glial cells. C's growth cone extends posteriorly and fasciculates with the same axon bundle as G's but about 10 h later in development. By the time C begins to extend predominantly in the posterior direction, there are several additional axon profiles in the bundle including X1 and X2 (Fig. 8D). The A/P fascicle continues to expand in size as more axons fasciculate with it.

This pattern of selective fasciculation, coupled with our knowledge of cell lineages, leads to two simple observations. First, cells from the same precursor fasciculate in different axon bundles. Second, axon bundles contain cells from different precursors. For example, just after G joins the A/P fascicle, it contains two axons (the As) from one precursor, two axons (the Ps) from a second precursor, and one axon (G) from a third precursor. Shortly thereafter, they are joined by two other axons (the Xs) from yet another precursor. Other progeny from these same precursors join other fascicles, as exemplified by G's siblings. Thus, the axons of unrelated neurones of diverse embryonic origin demonstrate a high affinity for one another, giving rise to their specific patterns of selective fasciculation.

SELECTIVE FASCICULATION BY FILOPODIAL ADHESION

There is a particular organization in the A/P fascicle. The relative apposition of the axons we observe in many different embryos suggests that A axons adhere tightly to other A axons, P axons to P axons, but A axons not as tightly to P axons and *vice versa*. Within the fascicle, the two tightly apposed P axons can be located anywhere around the perimeter of the two A axons. The tip of G's growth cone is always closely associated with the P and not the A axons (Fig. 8C), irrespective of the relative orientation of the four axons within the A/P fascicle. This finding suggests that not only is G able to distinguish the A/P fascicle from other axon bundles, but moreover is able to distinguish the P axons from the A axons within this fascicle (Raper *et al.* 1983*c*; Bastiani *et al.* 1984; Goodman *et al.* 1984).

G first recognizes the A/P fascicle, and specifically the P axons, *via* its filopodia. Differential filopodial adhesion appears to be the mechanism of cell recognition at these early embryonic stages and underlies the specific patterns of selective fasciculation. Thus, before considering the behaviour of G's filopodia, it is important to review briefly what is known about filopodia, most of which has been learned from *in vitro* studies.

Growth cones radiate many filopodia (approx. 0·1 μm in diameter, up to 50 μm in length) which transiently explore their environment. Many of these filopodia contact other cell surfaces. To some of these surfaces they strongly adhere, and to others their adhesion is much weaker. If adhesion is weak, during the contractile cycle, the filopodium is retracted; if however, its adhesion is strong, then tension in that direction is increased during the contractile cycle and the leading tip of the growth cone advances towards the point of attachment (Bray, 1982; Letourneau, 1982). Thus, filopodia test the adhesiveness of the available substratum within their grasp. Those filopodia with strong adhesion to other cells or surfaces will more effectively guide the growth cone during the contractile phase.

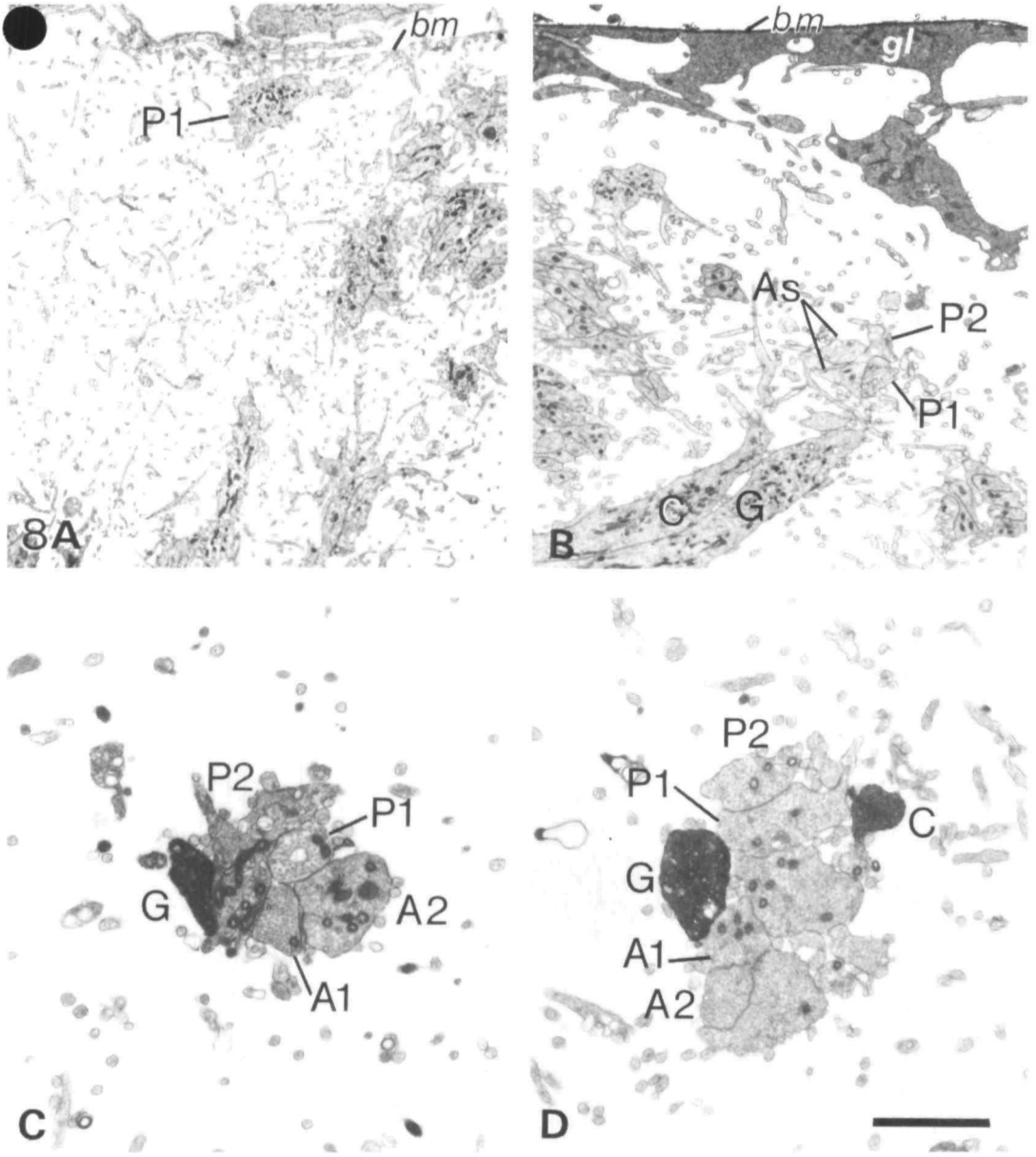


Fig. 8. The embryonic development of the A/P fascicle, from its pioneering by the P1 growth cone (A), to the first four axons (B), to the addition of G (C), and to the subsequent addition of other axons, as shown in this series of electron micrographs of the fascicle at 37 % (A), 39 % (B), 40 % (C) and 41 % (D) of development. (A) The P1 growth cone extends posteriorly along the dorsal basement membrane (*bm*) until it meets the anteriorly extending A1 growth cone. The P and the A growth cones fasciculate together and drop off the basement membrane. (B) G and C are extending across the posterior commissure from the left. Their growth cones are just ventral and medial to the A/P fascicle which has just formed and contains four axons: A1, A2, P1 and P2. Notice that the G and C growth cones are not in direct contact with any other axons; they are suspended by their filopodia which wrap around the P axons (not shown). (C) The characteristic close apposition of the tip of the G growth cone with the P axons. The G cell was filled with horseradish peroxidase (HRP). (D) The A/P fascicle after C and several other axons have joined it. Both G and C have been filled with HRP. In contrast to the G growth cone, the G axon at this later stage of development (41 %) shows no dramatic preference for the Ps *versus* the As. Several other axons have already added into the fascicle by this time. *gl*, glia; scale bar, A,B: 1·5 μm; C,D: 1 μm.

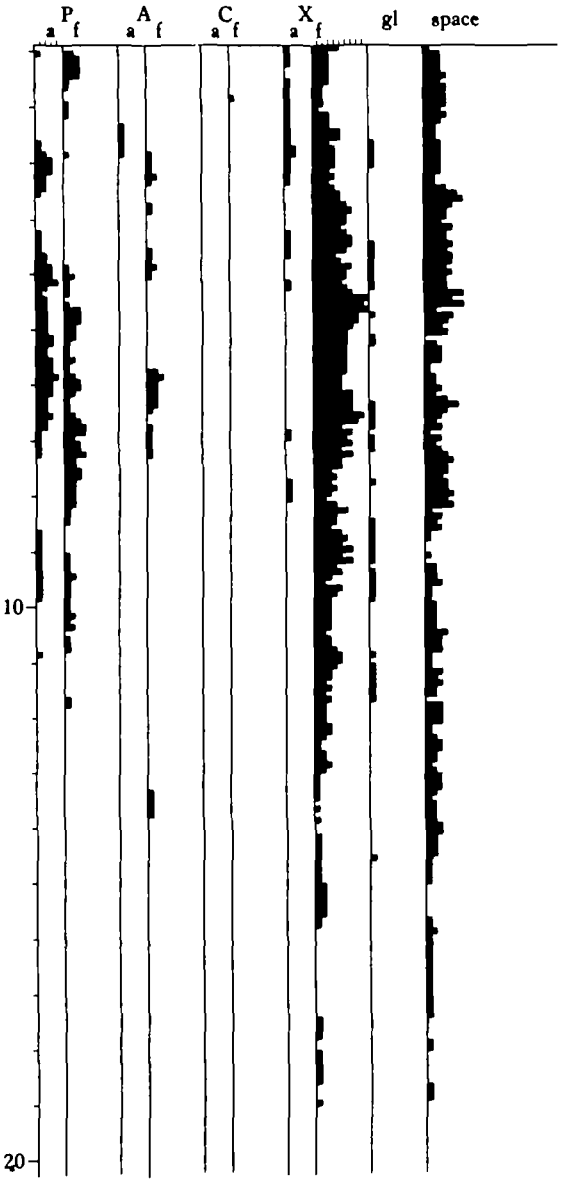


Fig. 9. Contacts by the G filopodia at a stage (39 % of development) before the G growth cone climbs onto the A/P fascicle. Schematic representation of the serial section reconstruction of the G and C growth cones and filopodia, and the cells and filopodia of the A/P fascicle. This shows the relationship between the filopodia extending from the anterior 5 μm of the G growth cone and the P cells (Pa,f), A cells (Aa,f), C cells (Ca,f), unidentified cells (Xa,f), glial cells (gl) and space. a and f represent the axons and filopodia respectively of the P, A, C and X cells. X represents the ~100 other axons and growth cones in the neuropile within filopodial grasp. The G filopodia are not in contact with any cells or other filopodia for a significant portion of their length (space). There is only minor contact with glia cells (gl) and other unidentified axons (Xa), but many with unidentified filopodia (Xf). The small number of contacts with the C growth cone is not surprising given the fact that C is behind and growing upon the G growth cone. The most significant comparison is between the A cells (Aa,f) and the P cells (Pa,f). The G cell filopodia have equal access to both As and Ps, yet they show a dramatic preference for the Ps. Compare Pa *vs* Aa, and Pf *vs* Af. Vertical scale is distance in microns, 1 μm/division (10 sections). Horizontal scale is number of filopodial cross sections, 1 filopodium/division.

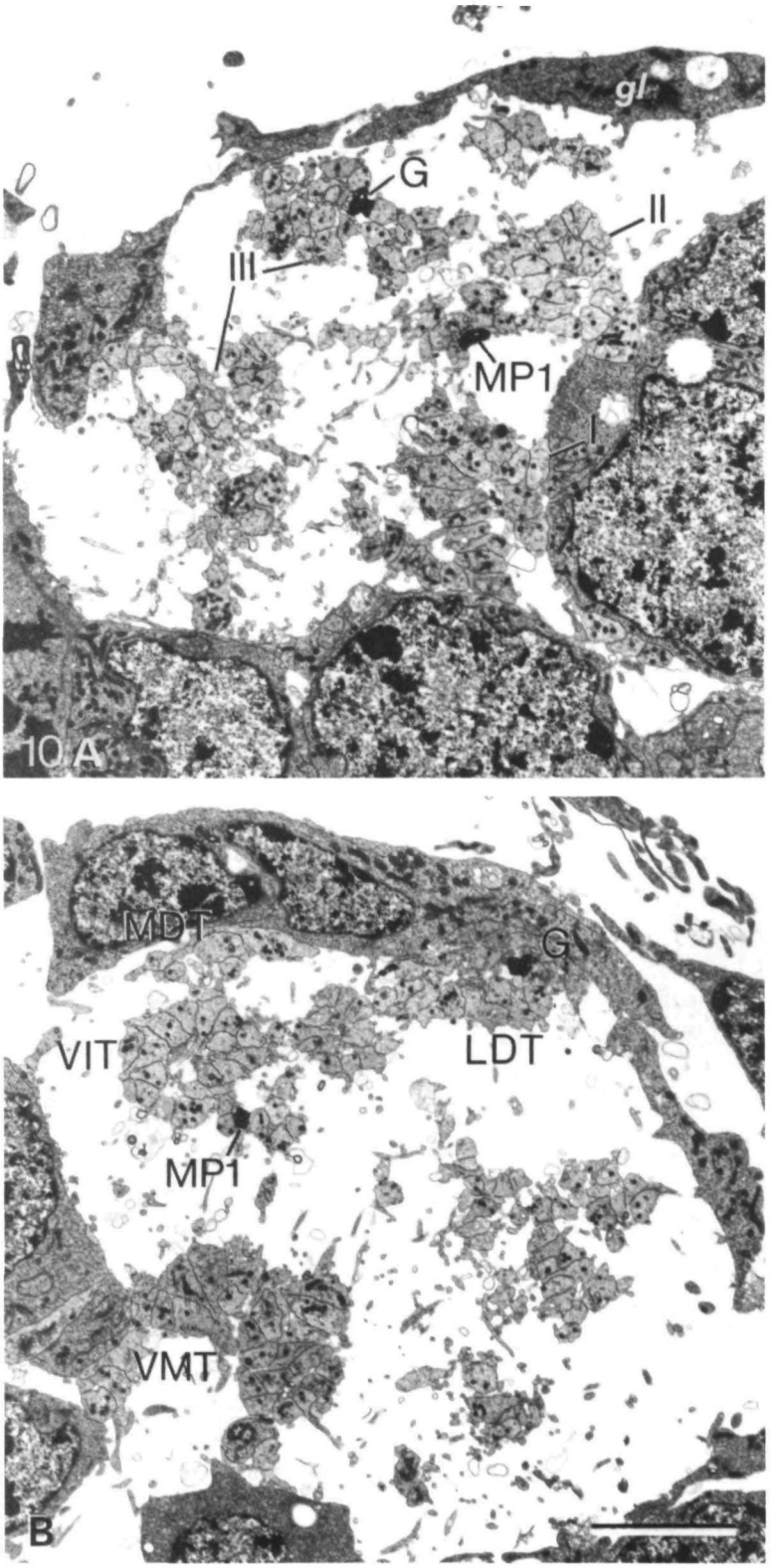
We reconstructed G's filopodia and their contacts in the TEM just before G climbed onto the A/P fascicle. At this time, the G and C growth cones had left the Q1 commissural fascicle containing their sibling's axons and had extended dorsally and laterally towards the A/P fascicle, appearing to be guided by strong filopodial and lamellipodial adhesion with the P axons (Fig. 8B). In fact, the leading filopodia and lamellipodia from G's growth cone appear to have wrapped around the P axons. The serial reconstruction demonstrates a dramatic overall preference of the G filopodia for the P axons instead of the A axons (Fig. 9) (Bastiani *et al.* 1984). This preference also extends to the P filopodia over the A filopodia (compare Pa,f to Aa,f). In addition, some of G's filopodia contacted glia, some were in space, and some contacted the axons or filopodia of unidentified neurones representing 100 or so different axons within filopodial grasp.

It appears that within the tangled spaghetti of filopodia in neuropilar space, G's filopodia can recognize the P filopodia just as they do the P axons. This implies that the recognition labels on embryonic neurones are expressed on their axons, growth cones and filopodia. Furthermore, it implies that at these early stages of development before the neuropilar terrain has become too densely woven, neurones are able to recognize one another at a great distance by filopodia contacting filopodia. Thus, many neurones are in contact with one another while the early patterns of selective fasciculation are being established. Later in development, when specific synaptic connections are formed, it is likely that the greater density of processes and reduced availability of space for filopodial exploration reduces the distances over which neurones can contact one another. Presumably, this limits a neurone's potential synaptic partners to those processes in its immediate neighbourhood.

Our results show that G's filopodia can distinguish the A/P fascicle from other axon bundles, and moreover within this fascicle can distinguish the P from the A axons (Bastiani *et al.* 1984). Although the electron micrographs present a static picture of a dynamic process, the results strongly suggest that this selective fasciculation is mediated by differential adhesion of the filopodia of the G growth cone to the A/P fascicle, and in particular, to the P axons.

Ablation experiments support these conclusions (Raper *et al.* 1984). When the A and P axons are ablated, the G growth cone behaves abnormally for the next 24 h, branching wildly throughout the neuropile, and does not show a high affinity for any other axon fascicle. When the A axons are ablated, the G growth cone behaves normally, fasciculating on the P axons. When the P axons alone are ablated, the G growth cone appears in general to ignore the A axons and behave abnormally, branching throughout the neuropile without high affinity for any other axon surface.

These examples of exquisite specificity have convinced us that many different molecules are differentially expressed on the surfaces of embryonic axon fascicles, or subsets of axons within them, and that they guide growing neurones to their appropriate targets by the selective adhesion of their filopodia to these labelled axonal pathways (Goodman *et al.* 1984). Monoclonal antibodies generated against the grasshopper neuroepithelium have revealed cell surface antigens whose temporal and spatial expression in the embryo correlate with the predictions of the cellular studies, namely, neurones whose axons fasciculate together share common surface antigens (Kotrla & Goodman, 1984).


gl
G
II
III
MP1
I
10 A
MDT
G
VIT
LDT
MP1
VMT
B

DEVELOPMENT OF THE CONNECTIVE

At the posterior end of the segment where the connective develops, at ~35 % of development the first three longitudinal axon fascicles contain only six axons, as described earlier: the vMP2 fascicle, the MP1/dMP2 fascicle, and the U fascicle with the U1, U2 and aCC axons (Fig. 4B) (the pCC from the next posterior segment is extending anteriorly along the MP1/dMP2 fascicle and has not yet reached this location). Initially all three fascicles are spatially separate within a space bounded on its dorsal side by the basement membrane and on its other sides by neurones and epidermal cells. The vMP2 fascicle forms along the inside, ventral surface; the MP1/dMP2 fascicle along the middle, dorsal surface; and the U fascicle along the outer, dorsal surface of this space.

In contrast to the embryonic neuropile, where many additional longitudinal axon fascicles develop between 35 % and 42 % of development, in the embryonic connective the additional axons simply add to the three existing fascicles, expanding them into much larger bundles that we call superfascicles I, II and III. These superfascicles begin to merge into each other and form an 'L' shaped connective by ~42 % of development (Fig. 4C). The results suggest that the three initial fascicles (vMP2, MP1/dMP2, U) are contained respectively within the three later superfascicles (I, II, III). The identity of the MP1/dMP2 fascicle within superfascicle II was confirmed by HRP injection of the MP1 neurone at this later time (Fig. 4C).

RELATIONSHIP OF NEUROPILE TO CONNECTIVE

How do the ~25 longitudinal axon fascicles per hemisegment in the embryonic neuropile become reduced to the three large superfascicles in the connective? To answer this question, we examined cross sections of the region anterior to the neuropile where the longitudinal fascicles begin to condense into the connective (Fig. 10). At 42 % of development, the left and right connectives in this embryo contained 136 and 140 axons respectively. At this intermediate level between the neuropile and connective, the ~25 neuropile fascicles are associated into 8–10 larger fascicles. By the time they reach the connective, these 8–10 larger fascicles associate into the three superfascicles. The vMP2 fascicle is within superfascicle I, the MP1/dMP2 fascicle is within II, and the A/P fascicle is within III.

It is within the neuropile that growth cones make their specific selective fasciculation choices, and ultimately their specific synaptic connections, and thus appropriately it is within the neuropile that many different spatially separated axon fascicles develop.

Fig. 10. Electron micrographs showing cross sections of the left (A) and right (B) longitudinal axon fascicles at the level just anterior to the A commissure (see Fig. 5), and just before the level where the connective forms. (A) The ~25 longitudinal axon fascicles in the neuropile at this level had combined into 8–10 larger fascicles which in the connective associate into the three superfascicles as labelled: I, II and III (see Fig. 4). (B) The major fascicles at this level can be identified according to the adult tracts they become: ventral medial tract (VMT; containing the vMP2 fascicle); ventral intermediate tract (VIT; containing the MP1/dMP2 fascicle including the MP1 axon); medial dorsal tract (MDT) and the lateral dorsal tract (LDT; containing the A/P fascicle including the G axon). The G and MP1 neurones have been filled bilaterally to verify the identity of these fascicles. *gl*, glia; scale bar, 10 μm.

Furthermore, each of these fascicles may have additional subdivisions of specific axon affinities within them. For example, the filopodia of the G growth cone specifically adhere to the P and not the A axons in the A/P fascicle.

The spatial separation of the developing fascicles within the large space of the embryonic neuropile is fortuitous for the investigator, and presumably equally helpful for growth cones since it allows easy access by their filopodia to many different axon surfaces. The specific clustering of the ~25 neuropile fascicles into 8–10 larger fascicles and then the three connective superfascicles suggests some affinity that particular fascicles have for one another. This affinity is likely to be lower than that shown by particular axons for one another within a fascicle, yet nevertheless it appears to bind certain fascicles together in a stereotyped pattern as they enter the connectives. Although individual fascicles appear to have specific labels, the clustering of particular fascicles suggests some common affinity amongst them. This may come about by some common lower affinity amongst groups of specific high affinity labels, or alternatively, different more widely distributed labels in addition to the specific ones.

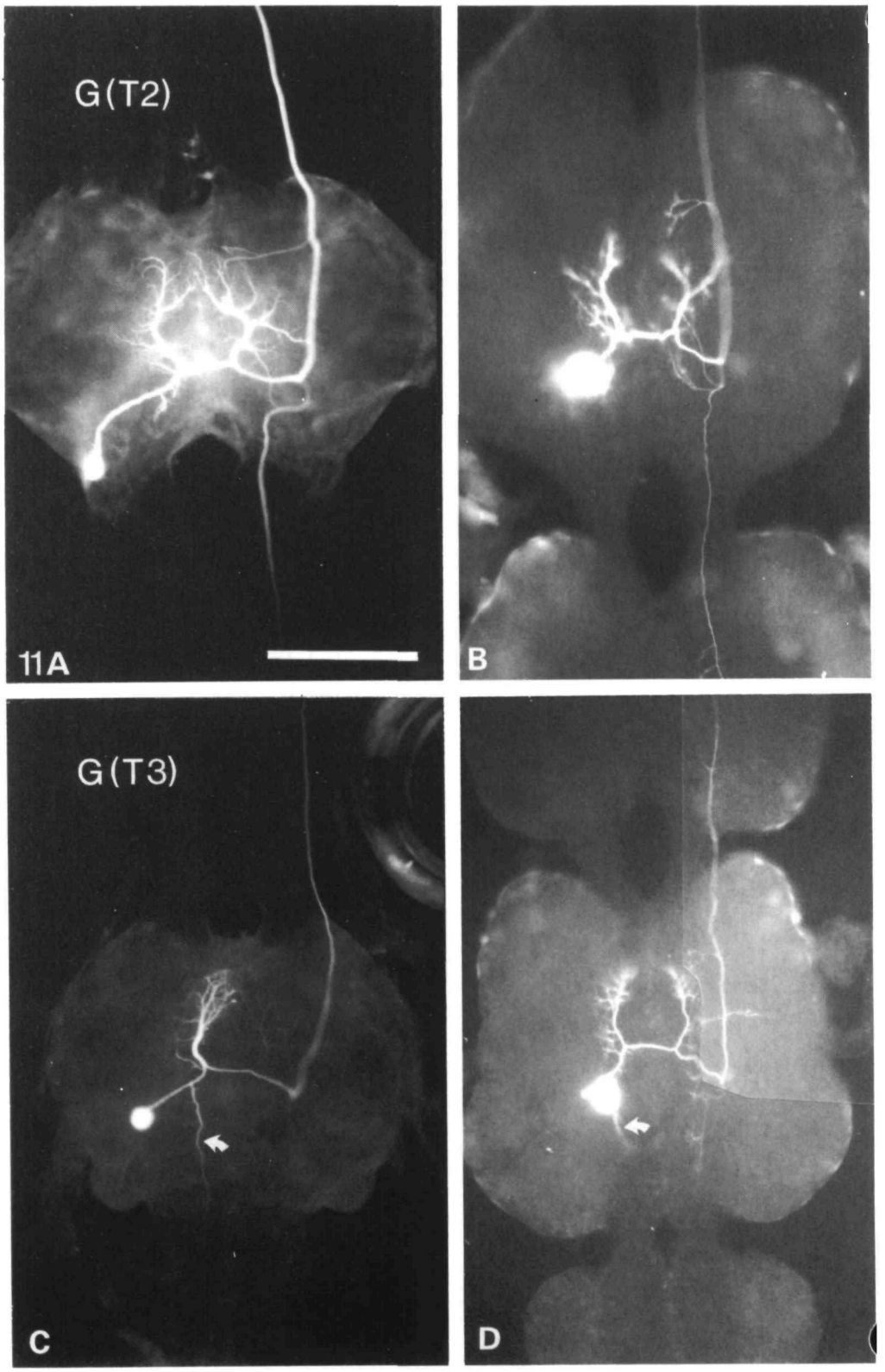
RELATIONSHIP OF EMBRYONIC FASCICLES TO ADULT TRACTS

The intermediate clustering into 8–10 large fascicles reveals an organization reminiscent of the axon tracts seen in the adult by Tyrer & Gregory (1982). We were initially puzzled that the embryo had ~25 longitudinal fascicles per hemisegment whereas the adult had only nine tracts. We presume that as the embryonic fascicles enlarged, many of them merge into one another to form the adult tracts. Much of the basic organization seen in the adult is apparent in the embryo at this intermediate level of clustering between the neuropile and connectives. At 42% of development we can identify the LDT, MDT, VIT and VMT tracts. Many other axons are present which are likely to represent the embryonic origins of the other tracts such as the DIT and VLT; these tracts, however, require further identification in the embryo.

The development and clustering of the embryonic fascicles leads to the following conclusions. The P1 growth cone pioneers the A/P fascicle which becomes part of the LDT (lateral dorsal tract). This is further substantiated by the location of the G axon in the embryonic A/P fascicle and in the adult LDT. The vMP2 growth cone pioneers the vMP2 fascicle which becomes part of the VMT (ventral median tract). The MP1 and dMP2 growth cones pioneer the MP1/dMP2 fascicle which becomes part of the VIT (ventral intermediate tract). Although we do not know the original pioneers and members for the other tracts, potentially each can be traced to its fascicle of embryonic origin.

Each of the adult tracts, however, clearly contains several separate embryonic fascicles. Furthermore, each embryonic fascicle may contain subsets of axonal associations. Thus, there is a much higher degree of axonal association beyond the nine adult tracts, and these associations are more easily revealed in the embryo where the neuropile fascicles are spatially distinct.

For example, when we compare the organization of the VIT at 42% of development at the intermediate level between neuropile and connective (Fig. 10), we find a symmetrical pattern of two groups of axons. On the left side, the VIT contains a rosette of 17 axons and an attached ventro-lateral cluster of nine axons (including MP1), and


G(T2)
11A
B
G(T3)
C
D

the right side, it contains a rosette of 18 axons and an attached ventro-lateral cluster of eight axons (including MP1). Each of these two subdivisions is likely to have yet further subdivisions of axonal associations.

SEGMENTAL HOMOLOGUES

Thus far we have presented evidence that a stereotyped orthogonal array of axon fascicles develops in each segment, and that growth cones faithfully fasciculate with specific axons in this array according to the neurone's lineage and previous interactions. This model is attractive because it helps explain development of neuronal specificity in a single segment by a series of cell recognition decisions based on the surface affinities of embryonic axons and dendrites. Furthermore, it suggests that these affinities change temporally, spatially or both for a single neurone during development as the primary growth cone makes its choices, and as subsequent growth cones make their choices.

Whereas the identified neurones in a single segment are dedicated for specific circuits that underly particular behaviour patterns, we wondered how the identified neurones in different segments in the same embryo become specialized in their morphology and physiology for different behavioural circuits. For example, every segment in the grasshopper embryo has an NB 7-4 which produces a G neurone, and yet these neurones are likely to do different things in different segments. Furthermore, we recently showed that different insects, from grasshoppers to moths to fruitflies, have G neurones (Thomas, Bastiani, Bate & Goodman, 1984), and yet they too are probably doing different things in different species. How do segmentally homologous neurones differentiate in segment-specific ways?

We examined the differentiation of the G neurone in five different segments of the grasshopper embryo (T1, T2, T3, A1 and A2), its morphology and some of its synaptic connections in three different segments of the adult grasshopper (T2, T3 and A1). Fig. 11 shows Lucifer Yellow injections of G(T2) and G(T3) in the adult and 60% developed embryo. Fig. 12 compares the morphology of the G neurone in segments T1–A2 in the 60% developed embryo.

In all five segments, G's primary axonal and dendritic growth cones selectively fasciculate in the same axon bundles. The basic structure of the G neurone remains unchanged. However, in T3, an additional posterior dendrite develops on the ipsilateral side, and in A2, the primary contralateral dendrite does not fully develop. In T2, a secondary axonal branch extends posteriorly in the connective to the next segment, whereas in many other segments, this branch merely develops into a local arborization. Thus, with these few variations on a theme, the basic morphology of the

Fig. 11. The morphology of the G neurone in the adult (A,C) and 60% developed embryo (B,D) in the mesothoracic (A,B) and metathoracic (C,D) ganglia. All neurones were filled with Lucifer Yellow. Much of the final adult morphology is already present by 60% development, although the relative dimensions of some processes have not yet completely formed (e.g. the contralateral dendrite of G in T3 is relatively much thinner than the ipsilateral dendrite in the adult than in the embryo). Note the secondary posterior axon extending to the next posterior segment on the contralateral side of G(T2) in the adult and the embryo, whereas this secondary axon is represented by only a short arborizing process in G(T3). G(T3) has an additional posterior dendrite on the ipsilateral side (arrows). Scale bar, A,B; 400 μm, C, 100 μm, D, 130 μm.

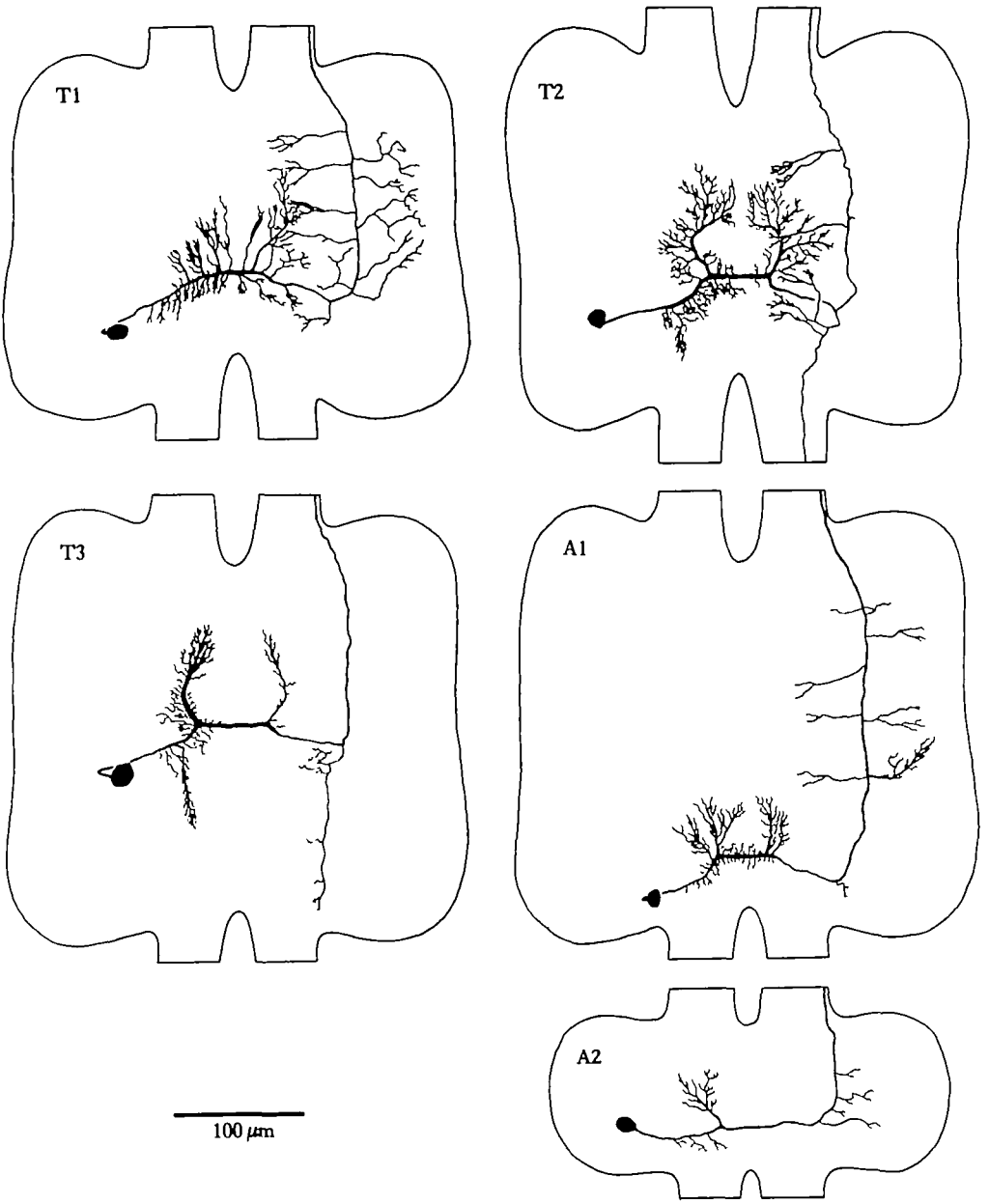


Fig. 12. The segmental homologues (lineally equivalent) of the G neurone at 60% of development in the prothoracic (T1), mesothoracic (T2), metathoracic (T3), first abdominal (A1) and second abdominal (A2) ganglia. The basic structure of the G neurone is the same in all segments; the primary axon fasciculates in the A/P fascicle (LDT tract in the adult) and the primary dendrites fasciculate in the VIT tract. However, there are also distinct segmental differences in the morphology. For example, G(T2) has a secondary axon extending posteriorly into the next segment, while in most other segments this branch arborizes locally. G(T3) extends a major ipsilateral dendrite posteriorly; this dendrite appears quite small in G(T2) and is not present at all in the other ganglia. In G(A2) the contralateral dendrite present in all other segments does not develop. Drawings made from wholemounts of Lucifer Yellow filled embryonic neurones.

G neurone remains constant because its primary axon and dendrites fasciculate in stereotyped bundles from segment to segment.

However, within the neighbourhoods chosen by the selective fasciculation of the G axon and dendrites, the fine branching pattern of the G neurone varies dramatically from segment to segment, presumably reflecting the segment-specific differences in synaptic connections. We have only a little information about the synaptic inputs and outputs of the G neurone in T2, T3 and A1, but this data demonstrates segment-specific differences in synaptic connections (Fig. 13). For example, the DCMD interneurone has access to the same dendritic branches of G(T2), G(T3) and G(A1), and yet DCMD bypasses G(T3) and only connects to G(T2) and G(A1). This suggests that segment-specific synaptic connections are made by the G neurone as it interacts with only certain subsets of neurones within the neighbourhood established by its pattern of selective fasciculation.

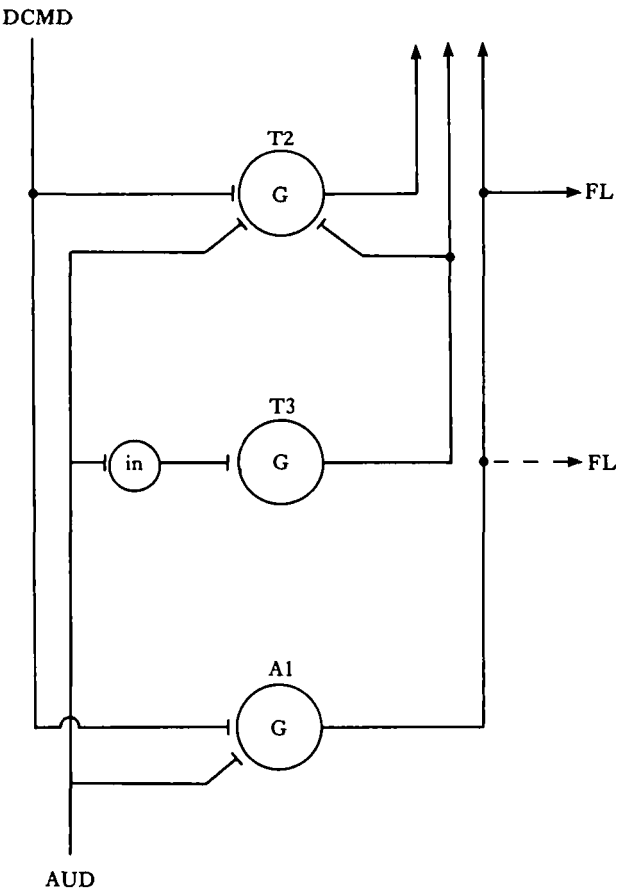


Fig. 13. Segment-specific differences in synaptic connections of the G neurone and its homologues in the T2, T3 and A1 ganglia. The descending contralateral movement detector interneurone (DCMD) sends its axon posterior from the brain. DCMD synapses on the G neurone in T2, bypasses G in T3, and then synapses again with G in A1, although all three Gs have their dendrites in the same region of neuropile. Primary auditory afferents (AUD) entering in the auditory nerve make monosynaptic connections with G(A1) and G(T2), but only polysynaptic connections *via* an interneurone with G(T3). G(A1) synapses directly with flight motor neurones (FL) in the mesothoracic and possibly metathoracic ganglia, in contrast to G(T2) and G(T3). Finally, G(T3) synapses onto G(T2), whereas G(A1) does not synapse on either of the more anterior G neurones.

GROWING UP IN THE RIGHT NEIGHBOURHOOD

Evolutionary plasticity allows the G neurone in different segments of the same species, or in the same segment of different species, to make different patterns of synaptic connections. But it appears as if evolution has left the fundamental pattern untouched. The orthogonal scaffold of axon fascicles has remained constant throughout the evolution of different segments and different insects, as have the patterns of selective fasciculation by the primary axons and dendrites. To tamper with this basic plan would probably have resulted in scrambled circuits rather than finely retuned connections. Evolution appears to have generated its synaptic variations within this common framework by tampering with the later recognition events within neighbourhoods rather than the initial events establishing them. Given a neurone's dendritic and axonal neighbourhoods, evolution has in general played with the local events that occur after selective fasciculation, namely, the specific choices of synaptic partners within the local neighbourhood.

Thus, we conclude that neuronal specificity is based largely on a series of cell recognition events that first generate specific neighbourhoods by the patterns of selective fasciculation, and subsequently generate specific connectivity by the patterns of synaptic partners within these neighbourhoods. The notion emerging from these studies is that a basic segmentally-repeated pattern arises during embryogenesis: a stereotyped axonal scaffold upon which growth cones faithfully fasciculate. Evolutionary plasticity allows for the specialization of lineally equivalent neurones in different segments or different species by changes in their synaptic partners within the context of their neuropilar neighbourhood. As a consequence of their earlier selective fasciculation choices, their neighbourhood presents them with a particular subset of possible partners.

These ideas are in many respects remarkably similar to conclusions based on serial reconstructions of the nervous system of the nematode (White, Southgate, Thomson & Brenner, 1983). The relatively one-dimensional ventral nerve cord of *Caenorhabditis elegans* is organized into a single longitudinal tract containing bundles of axons that in general do not branch. Within the neighbourhoods established by these axon associations, synaptic contacts are made with a subset of adjacent axons. White *et al.* (1983) conclude that synaptic connectivity is likely to be coordinated by intrinsic specificities, process guidance and process placement, notions similar to those used here to describe neuronal specificity in the grasshopper.

It is heartening to think that in nematodes and insects, the only two animals thus far analysed at this detailed cellular level, the same cellular and molecular mechanisms appear to be at work in the generation of neuronal specificity. It makes us think that these mechanisms are likely to have been conserved throughout evolution, and that the nervous systems of higher animals may simply use more complex versions of these same mechanisms.

We thank Fran Thomas and Yen Tang for technical assistance. MJB is supported by a Developmental Biology Training Grant from the NICHHD, KJP is supported by the Canadian Medical Research Council, and CSG is supported by grants from the N.S.F., N.I.H. and March-of-Dimes Birth Defects Foundation.

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