Cell Recognition During Neuronal Development

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Summary. Insect embryos, with their relatively simple nervous systems, provide a model system with which to study the cellular and molecular mechanisms underlying cell recognition during neuronal development. Such an approach can take advantage of the accessible cells of the grasshopper embryo and the accessible genes of *Drosophila*. The growth cones of identified neurons express selective affinities for specific axonal surfaces; such specificities give rise to the stereotyped patterns of selective fasciculation common to both species. These and other results suggest that early in development cell lineage and cell interactions lead to the differential expression of cell recognition molecules on the surfaces of small subsets of embryonic neurons whose axons selectively fasciculate with one another. Monoclonal antibodies reveal surface molecules in the *Drosophila* embryo whose expression correlates with this prediction. It should now be possible to isolate the genes encoding these potential cell recognition molecules and to test their function through the use of molecular genetic approaches in *Drosophila*.

The human nervous system is composed of hundreds of billions of nerve cells, each with extensive processes that intertwine and synaptically interconnect in a highly specific way. How the nervous system is properly "wired" during development, that is, how neurons recognize one another and make the appropriate synaptic connections, is one of the major questions in modern biology.

This question was first addressed by Cajal, who described the ameboid processes at the end of embryonic axons which he called growth cones (*1*). Harrison confirmed Cajal's axonal outgrowth hypothesis by describing the extension of growth cones from living frog spinal cord explants in tissue culture (*2*). Cajal and Harrison both observed that the outgrowth of axonal processes was not random, but rather growth cones extended along stereotyped pathways to find and recognize their correct targets. Furthermore, they both hypothesized that growth cones must be endowed with some exquisite chemical sensitivity and their targets chemically specified to allow them to make their correct choices during development.

This idea was further elaborated by Sperry, who proposed the chemoaffinity hypothesis to explain how growth cones from retinal ganglion cells find their correct targets in the optic tectum during regeneration in amphibians (*3*). Sperry concluded that "the final course laid down by any given fiber reflects the history of a continuous series of decisions based on differential affinities between the various advance filaments [today called filopodia] that probe the surroundings ahead and the diverse elements that each encounters" (*3*, p. 707).

Over the past decade, much has been learned about the structure and behavior of growth cones by studies on dissociated neurons in tissue culture (*4–6*). Growth cones extend many finger-like filopodia, which are about 0.1 μm in diameter and typically about 30 μm long. These filopodia radiate in many directions from the growth cone, transiently exploring their environment. Filopodia are dynamic structures, extending in length, moving about, and retracting in a matter of minutes. Most are short-lived and regress into the growth cone. However, some persist when they contact and differentially adhere to particular surfaces; these play a key role in guiding the growth cone (*7*).

How are neuronal growth cones guided to their specific targets in a developing embryo? To what extent are neuronal surfaces differentially labeled early in development, and to what degree can growth cones and filopodia specifically recognize these surface labels? What early events cause different neurons to express specific surface labels? And finally, what is the molecular basis of cell recognition during neuronal development?

To answer these questions at the level of individual cellular interactions and underlying molecular mechanisms, many of us who hope to someday understand how the human brain is wired during development study instead the simpler brains of invertebrate animals (*8*). Compared with the developing vertebrate brain, the neurons in the central nervous system (CNS) of insect embryos are large and few. The insect nervous system includes a chain of relatively simple segmental ganglia (reflecting the segmented body), each containing about 1000 pairs of neurons, many of which can be individually identified by their unique morphology and pattern of synaptic connections.

It is therefore possible to study how growth cones find and recognize their appropriate targets during development at the level of individual cells and potentially at the level of the individual recognition molecules. We have been carrying out such studies on the embryos of two different insects, one large (the grasshopper) and the other small (*Drosophila*), because of the advantages they offer, respectively, for a cellular and molecular genetic analysis of this problem.

Neuronal Recognition in the Grasshopper Embryo

Each developing segmental ganglion in the insect CNS arises from the neuroepithelium and consists of a ventral layer of neuronal precursor cells, a middle layer of cell bodies of their neuronal progeny, and a dorsal layer of neuronal processes. As each neuron differentiates, it extends a growth cone dorsally into the region called neuropil in which all of the axonal and dendritic processes intertwine and interconnect. The neuropil initially develops as an orthogonal scaffold of axon bundles organized into commissures that connect the two sides of a single segment, longitudinal tracts that connect neighboring segments, and nerves that connect the segmental ganglia to the periphery.

Neuronal specificity in insect embryos

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is largely achieved by a sequential series of cell recognition events, culminating in the formation of specific synaptic connections. By the time embryonic neurons begin forming synapses, however, most of the 1000 neurons per hemisegment have extended processes into the developing neuropil. Unfortunately, at these later stages of development, the developing neuropil in this seemingly simple nervous system is a complex tangle of neuronal processes, and thus is not appealing for a precise cellular analysis. If we examine earlier stages when only a handful of neurons have extended axonal processes, however, the system is simple enough for the analysis of the early events generating neuronal specificity: the selective affinities displayed by growth cones and their filopodia for the surfaces of other embryonic neurons.

Many of the earliest events of cell recognition in the developing CNS involve the specific choices made by growth cones as they extend onto particular axonal surfaces. Our results in the grasshopper embryo suggest that (i) the selective affinities of filopodia guide neuronal growth cones, and (ii) (just as in tissue culture) the differential adhesion of filopodia is likely to mediate these selective affinities. These differential affinities give rise to the stereotyped patterns of axon bundles (or fascicles). Such selective fasciculation gives rise to the orthogonal scaffold of axon bundles (Fig. 1).

Table 1. Identified neurons and axon fascicles. Some designations are abbreviations (NB, neuroblast; A or a, anterior; P or p, posterior; CC, corner cell; VUM, ventral unpaired median; MP, midline precursor; d, dorsal; v, ventral; f, fascicle; IS, intersegmental); others are arbitrary.

Identified neurons		Identified axon fascicles	
Name	Mitotic ancestry	Name	Initial axons*
Neuroblast progeny			
G, C	Siblings, NB 7–4	A–P f.	A1, A2; P1, P2; G
A1, A2	Siblings	vMP2 f.	vMP2
P1, P2	Siblings	MP1–dMP2 f.	MP1; dMP2; pCC
aCC, pCC	Siblings, NB 1–1	U f.	U1, U2; aCC
U1, U2	Siblings	IS nerve	U1, U2; aCC; RP1, RP2; VUM1, VUM2
RP1, RP2	Siblings		
VUM1, VUM2	Siblings, MNB		
Midline precursor progeny			
MP1	Sibling is MP1 on other side		
dMP2, vMP2	Siblings, MP2		

*Other axons continue to join these fascicles during development. For example, the A–P f. is joined by P3, C, X1, and X2 (Fig. 1).

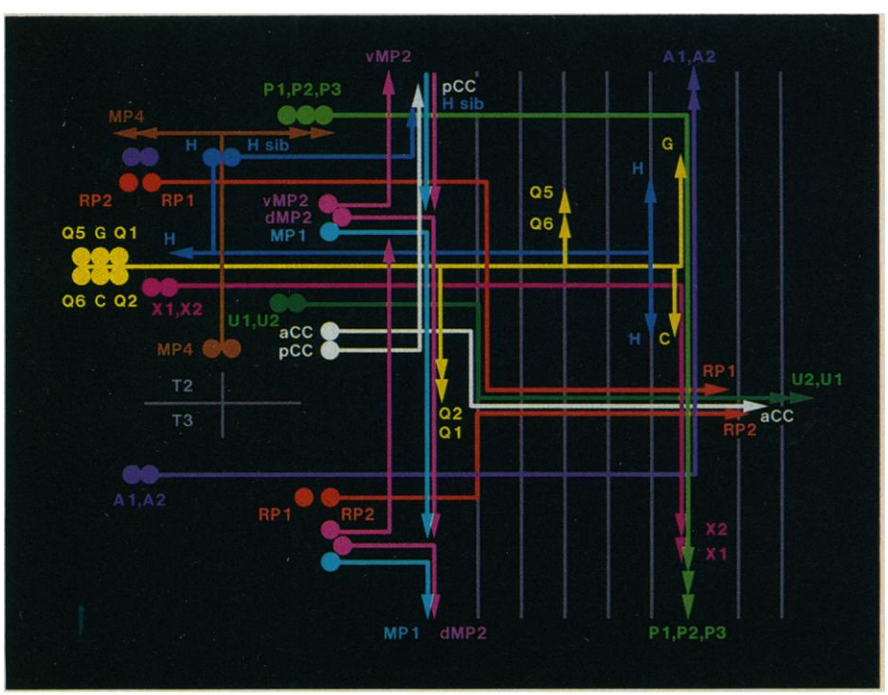

Fig. 1. Schematic diagram of selective fasciculation in the grasshopper embryo, showing the divergent and cell-specific choices made by the growth cones of identified neurons in one hemisegment. With minor differences, these patterns resemble those in the *Drosophila* embryo (*30*). At 40 percent of embryonic development, about 100 neurons have extended axons in each hemisegment, 26 of which are shown here. These 100 axons are organized into about 25 different longitudinal axon fascicles, 10 of which are shown here, as well as the intersegmental nerve (horizontal fascicle). Their axons selectively fasciculate with one another to form the orthogonal scaffold of axon bundles. Related neurons from the same lineage (shown by the same color), although confronted with the same environment, often diverge and choose different fascicles in the developing neuropil. At this stage of development, growth cones are typically within filopodial grasp of all of these longitudinal axon fascicles in one hemisegment. Those identified neurons discussed in the text are listed in Table 1.

Selective Fasciculation by the G Neuron: Test of the Labeled-Pathways Hypothesis

Many of our previous studies on neuronal recognition focused on the analysis and manipulation of a single identified neuron, the G neuron (Table 1), at a single choice point in the grasshopper embryo at 40 percent of development (*9–15*) (one of the yellow cells in Fig. 1). The G growth cone, like most other embryonic growth cones, finds itself surrounded by the axons of earlier differentiating neurons. At this stage, about 100 neurons in each hemisegment have extended axons; these 100 axons are organized into about 25 different longitudinal axon bundles (Fig. 1) (*12*).

The G growth cone radiates profuse tufts of filopodia that contact the surfaces of most of these 25 different longitudinal fascicles (*12*). Although the G growth cone has access to nearly all of the approximately 100 different axonal surfaces, it invariably chooses to fasciculate on a discrete bundle of four axons called the A-P fascicle (Table 1) in preference to all other axon bundles (*10–12*). At this early stage of development, the A-P fascicle contains the A1, A2, P1, and P2 axons; other axons continue to join this fascicle during development (Fig. 1). Extensive ultrastructural (*12*) and experimental (*13*) analysis demonstrates (i) that G is able to distinguish the A-P fascicle from all other longitudinal fascicles; and (ii) that within the A-P fascicle, G is able to distinguish the two P axons from the two A axons. For example, when the P axons are ablated, the G growth cone behaves abnormally and does not show a high affinity for any of the remaining 100 or so axons, including the A axons; when the A axons are ablated, the G growth cone behaves normally and fasciculates with the P axons

(*13*). The degree of neuronal specificity at this early stage of development is exquisite.

These studies of the selective fasciculation by the G growth cone led to the initial proposal (*10, 16*) and later experimental test (*11–14*) of the labeled-pathways hypothesis, which predicts that different neighboring axon fascicles in the embryonic neuropil are differentially labeled by surface recognition molecules. Growth cones use these labels to distinguish among those axon bundles within their filopodial grasp and select the appropriate fascicle. At 40 percent of development, the hypothesis predicts that the A-P fascicle, and in particular the P axons, have a unique surface label shared by none of the other 25 fascicles.

The aCC and pCC Neurons as a Simple Model System

We began studying neuronal recognition by a cellular analysis of the selective affinity of the G growth cone at 40 percent of development when only 100, rather than all 1000, neurons had extended processes. The embryonic neuropil at this stage is still very complex, however, and thus 100 axons organized into 25 fascicles is perhaps too many for the precise analysis that we envision. An alternative approach is to study earlier stages of development, when the first growth cones selectively fasciculate to form the first axon bundles.

Here we review our recent studies on an even simpler model system within this simple system: the earliest recognition events by fewer than ten neurons (*16–22*). The first three longitudinal axon fascicles in the grasshopper embryo initially contain the axons of seven identified neurons (Table 1 and Fig. 2). The growth cones of these first seven neurons are able to distinguish one another's surfaces, and by their specific interactions, selectively fasciculate with one another to form these three axon bundles (Fig. 2, B and C). We focus our attention on the selective affinities displayed by the growth cones of two of these cells, the sibling aCC and pCC neurons (*21, 22*).

Selective Affinities of the aCC and pCC Growth Cones

The aCC and pCC neurons are siblings (Table 1) that arise at the anterior edge of the segment posterior to the one they eventually reside in. They migrate about 100 μm anteriorly across the segment border to their final location. By the time they migrate across the border, they already begin displaying cell-specific behavior and presumably cell-specific surface labels. The leading edge of the pCC extends around the lateral edge of the aCC and points directly toward the posteriorly extending MP1 and dMP2 growth cones (30 percent in Fig. 3A). Extensive analysis by the light (*18*) and electron (*19*) microscope indicates that the MP1 filopodia preferentially contact the surface of the pCC cell body rather than the aCC, even though the MP1 filopodia have equal or better access to the aCC surface. This selective affinity of the filopodia from the MP1 growth cone suggests that the aCC and pCC have differentially labeled surfaces.

As the two sibling cells cease their migration and take up residence just posterior to the MP1 and dMP2 neurons (31 percent in Fig. 3A), their growth cones make divergent choices, ultimately fasciculating with different axon bundles. The pCC growth cone fasciculates with the MP1 and dMP2 axons. In contrast, the growth cone of the aCC remains relatively stationary. Although the filopodia of the pCC growth cone display a high affinity for the MP1 and dMP2 axons, the filopodia of the aCC growth cone do not (Fig. 3, A and C).

Only after 10 to 15 hours (33 percent in Fig. 3A) does the behavior of the aCC growth cone dramatically change; this change occurs precisely when the U1 and U2 growth cones appear on the dorsal surface within filopodial grasp (33 percent in Fig. 3, A and C). The U1 and U2 neurons extend growth cones that arrive at the dorsal basement membrane (Fig. 2B) and turn posteriorly to pioneer a third axon fascicle (Fig. 2, B and C; 33 to 35 percent in Fig. 3A). After extending

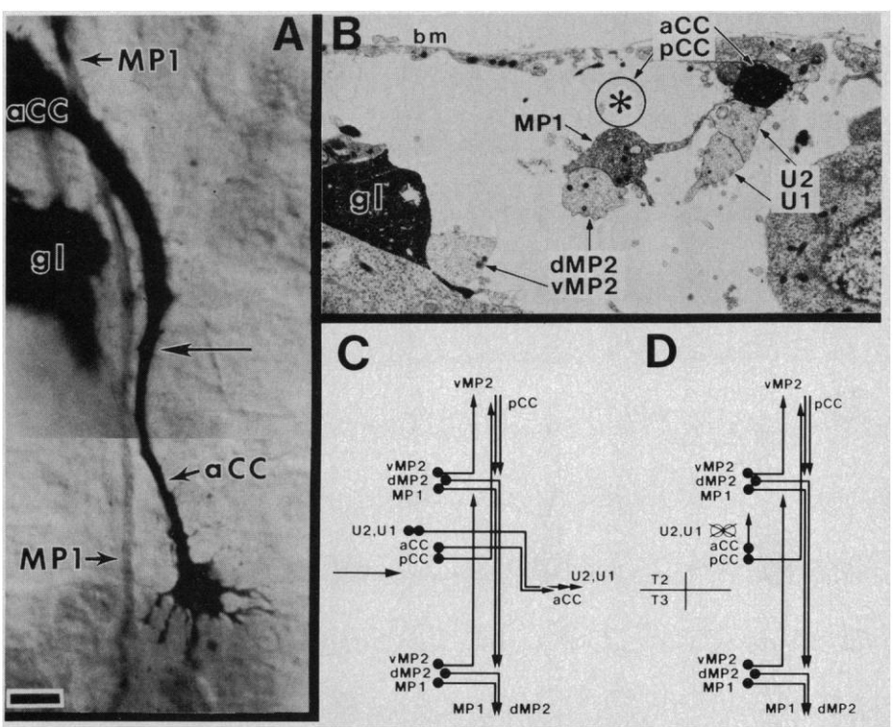

Fig. 2. Selective affinity of the pCC growth cone for the MP1-dMP2 fascicle, and of the aCC growth cone for the U fascicle (Table 1). Light (A) and electron (B) micrographs of horseradish peroxidase–filled neurons. Schematic diagrams (C and D) of the first three longitudinal axon fascicles (vMP2, MP1-dMP2, and U) in each hemisegment of the grasshopper embryo, and the seven identified neurons whose axons fasciculate to form these three bundles. (A) Dorsal light micrograph of the whole mount preparation [arrow notes location of section shown in (B)]. At this stage (35 percent) the aCC growth cone has just reached the segment border by extending posteriorly along the U fascicle; at the segment border, it is about to turn laterally along a large, epidermal cell called the segment boundary cell (not shown; see Fig. 4A for an electron micrograph of this preparation at the level of the growth cone). Abbreviation: gl, glia. Scale bar, 5 μm. (B) Transmission electron micrograph of the same preparation. The pCC axon extends anteriorly along the MP1-dMP2 fascicle and is not present in this plane of section (asterisk). Abbreviation: bm, basement membrane. Scale bar in (A) indicates 1 μm in (B). (C) Schematic diagram showing the selective affinities of the pCC and aCC growth cones. (D) Selectively ablating the U1 and U2 neurons prevents the posterior extension of the aCC, although such manipulations affect neither the formation of the vMP2 or MP1-dMP2 fascicles nor the normal choice of the pCC (*22, 23*).

posteriorly, the U's turn laterally at the segment boundary to pioneer the intersegmental nerve, seemingly guided by a large, conspicuous epidermal cell, called the segment boundary cell (Fig. 4A). Once the U's reach the basement membrane and begin extending posteriorly, the aCC growth cone changes its direction and extends laterally toward them. The aCC growth cone continues to extend posteriorly along the U fascicle (Fig. 2B). It then turns laterally along the same segment boundary cell (Fig. 4A) earlier followed by the U's.

The labeled-pathways hypothesis predicts that the first three fascicles are differentially labeled and that the aCC and pCC growth cones are differentially determined in their ability to make specific choices of which labeled pathway to follow. To test this hypothesis, we ablated the U1 and U2 neurons with a laser microbeam before they extended growth cones and before the aCC and pCC neurons had migrated to their final position (30 percent in Fig. 3A). Figure 3B shows the resulting morphology of the control and experimental aCC after approximately 5 percent of additional development in embryo culture (*13, 22, 23*). The control aCC extended along its normal posterior pathway. However, the growth cone of the experimental aCC continued to point anteriorly without choosing any particular pathway.

These results thus provide further support for the labeled-pathways hypothesis and suggest that early in development the first three longitudinal axon fascicles are differentially labeled on their surfaces. In the absence of the U's, the aCC growth cone appears uninterested in the other four axons (vMP2, MP1, dMP2, and pCC) and instead continues to point anteriorly (Figs. 2D and 3B). These results argue against (i) the simple location of the axons, (ii) subtle timing mechanisms, or (iii) simple quantitative differences in the expression of a common surface label being the major determinant. Rather, the aCC growth cone seems to show an absolute preference rather than a hierarchical one. These experiments support the notion that the surface of the U axons has some special distinguishing label that normally guides the aCC growth cone onto them and that is not expressed by the four other axons in the vicinity.

These results provide an example of specificity that, in addition to the example of the G growth cone and its selective affinity for the P axons within the A-P fascicle, convinces us that many different molecules or combinations of molecules are differentially expressed on the surfaces of these early embryonic axon fascicles or subsets of axons within them. These recognition molecules are likely to guide growing neurons to their appropriate targets by the selective affinity (most likely mediated by selective adhesion) of their filopodia.

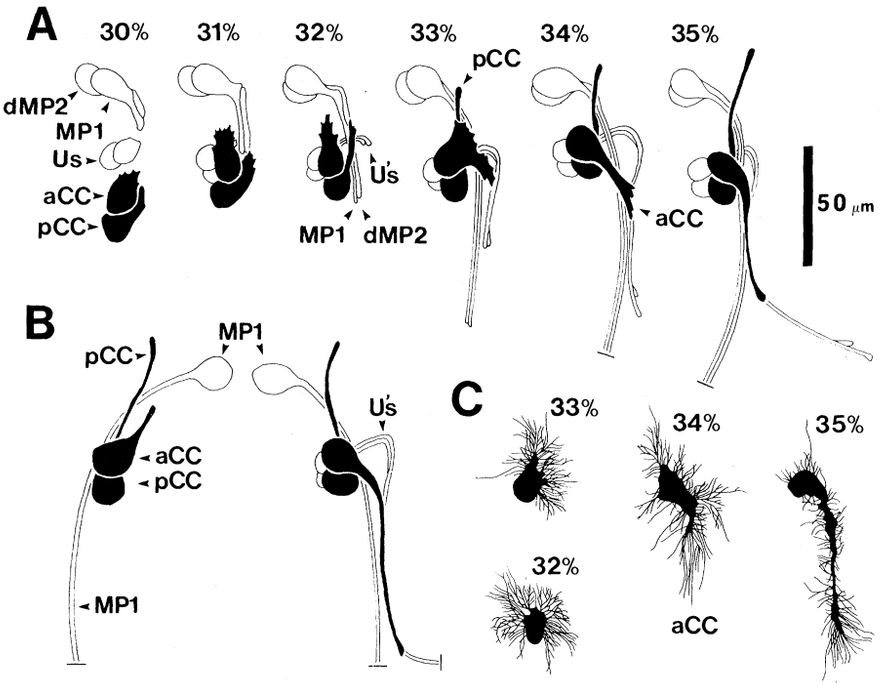

Fig. 3. Development of the pCC and aCC neurons in the grasshopper embryo, in normal (A and C) and experimental (B) embryos (*21, 22*). These camera lucida drawings show the aCC and pCC growth cones (A and B) and their filopodia (C) in relation to the MP1, dMP2, U1, and U2 axons. (A) Drawings of whole mount embryos stained with the I-5 Mab (*42*) between 30 and 35 percent of embryonic development, showing the axons as they fasciculate into two longitudinal bundles: the MP1-dMP2 and U fascicles. Not shown is the vMP2 axon, which extends anteriorly ventral and sometimes medial to the MP1-dMP2 fascicle (Fig. 2). (B) Experimental test of the labeled-pathways hypothesis. Camera lucida drawing of an embryo stained with I-5 Mab in which the U cell bodies in the left hemisegment were ablated with a laser microbeam at 30 percent of development; the embryo was then cultured for 48 hours to a stage equivalent to about 35 percent of development. (C) Camera lucida drawings of aCC neurons showing their filopodia for comparison to the positions of other axons (A). The filopodia were revealed by injecting the aCC with Lucifer yellow followed after fixation by an anti–Lucifer yellow antibody and horseradish peroxidase immunocytochemistry (*18*). Initially the cell body extends numerous filopodia, but as the growth cone extends, the filopodia disappear from the cell body and become more localized to the tip of the growth cone.

Cell Lineage and Cell Interactions Specify aCC and pCC Neurons

By the time the aCC and pCC neurons migrate across the segment border (and before they extend growth cones), these two sibling neurons already begin displaying cell-specific behavior and probably cell-specific surface labels. How does this come about?

The approximately 1000 pairs of neurons in each segment are generated during embryogenesis in the neuroepithelium from a stereotyped segmental pattern of neuronal precursor cells consisting of neuroblasts (NB's), which generate most of the neurons (*24*) and midline precursor cells, which generate only a few (*17*) (Fig. 5A). Each NB maintains its large size as a stem cell while it divides repeatedly to give rise to a chain of smaller cells, the ganglion mother cells (GMC's), each of which divides once more to generate a chain of doublets, which then differentiate into neurons. Each NB contributes a family of 6 to 100 neuronal progeny to the developing segmental ganglion before it undergoes programmed cell death.

Since the first demonstration in the grasshopper embryo that particular identified neurons are generated at specific branch points of NB family trees (*25*), other neuronal cell lineages have been described (for example, *9, 16, 23, 26*). The aCC and pCC neurons, for example, are siblings that arise from the first GMC from NB 1-1 (Table 1) (*18*). Does the mitotic ancestry of these cells actually determine some aspects of their unique

fate, or alternatively, is it their characteristic position and thus stereotyped interactions that impart their unique specificity? Selective ablations of identified cells at different stages of development have helped distinguish between these alternatives and have shown that both are in part correct.

The NB's develop out of groups of epidermal cells (EC's) in the neuroepithelium. Normally, NB 1-1 develops before its lateral neighbor, NB 1-2; at this stage, only EC's sit lateral to NB 1-1. When NB 1-1 is killed at this stage (before it has generated its first GMC), one of the neighboring EC's in the NB 1-2 position replaces it and enlarges into a NB that both occupies the NB 1-1 position and generates the identified neurons normally produced by NB 1-1 (Fig. 5C) (*27*). These results suggest that the EC's are not uniquely determined, but rather can assume different NB fates. Specification of EC's to become particular NB's seems to be determined by position (*27*).

After replacement of the ablated NB 1-1, the new NB 1-1 begins dividing, producing its first GMC with a delay of about 3 percent after the original NB 1-1 would normally have produced its first GMC. This delayed GMC gives rise to two neurons which then migrate anteriorly across the segment boundary, again with a temporal delay, and upon reaching their final position differentiate into the aCC and pCC neurons. Although they extend growth cones about 3 percent later than their normal counterparts, they make the same cell-specific fasciculation choices.

These results suggest that the lineage of the first GMC, rather than its spatiotemporal environment, may determine what progeny it will produce. To further test this notion, we ablated the first GMC and examined whether the second GMC would produce the aCC and pCC neurons. The progeny from the second GMC did not differentiate into the aCC and pCC neurons (Fig. 5D), even though they encountered the same spatiotemporal environment seen by the first GMC from the replaced NB 1-1 (Fig. 5C). Furthermore, the aCC and pCC neurons were not replaced by progeny of neighboring NB's. These results suggest that a neuron's mitotic ancestry (its NB and GMC of origin) plays a role in its determination (*27*).

Finally, we wondered what causes the aCC to differ from the pCC. The first GMC from NB 1-1 divides symmetrically to produce two progeny, yet by the time they migrate anteriorly, they behave dif-

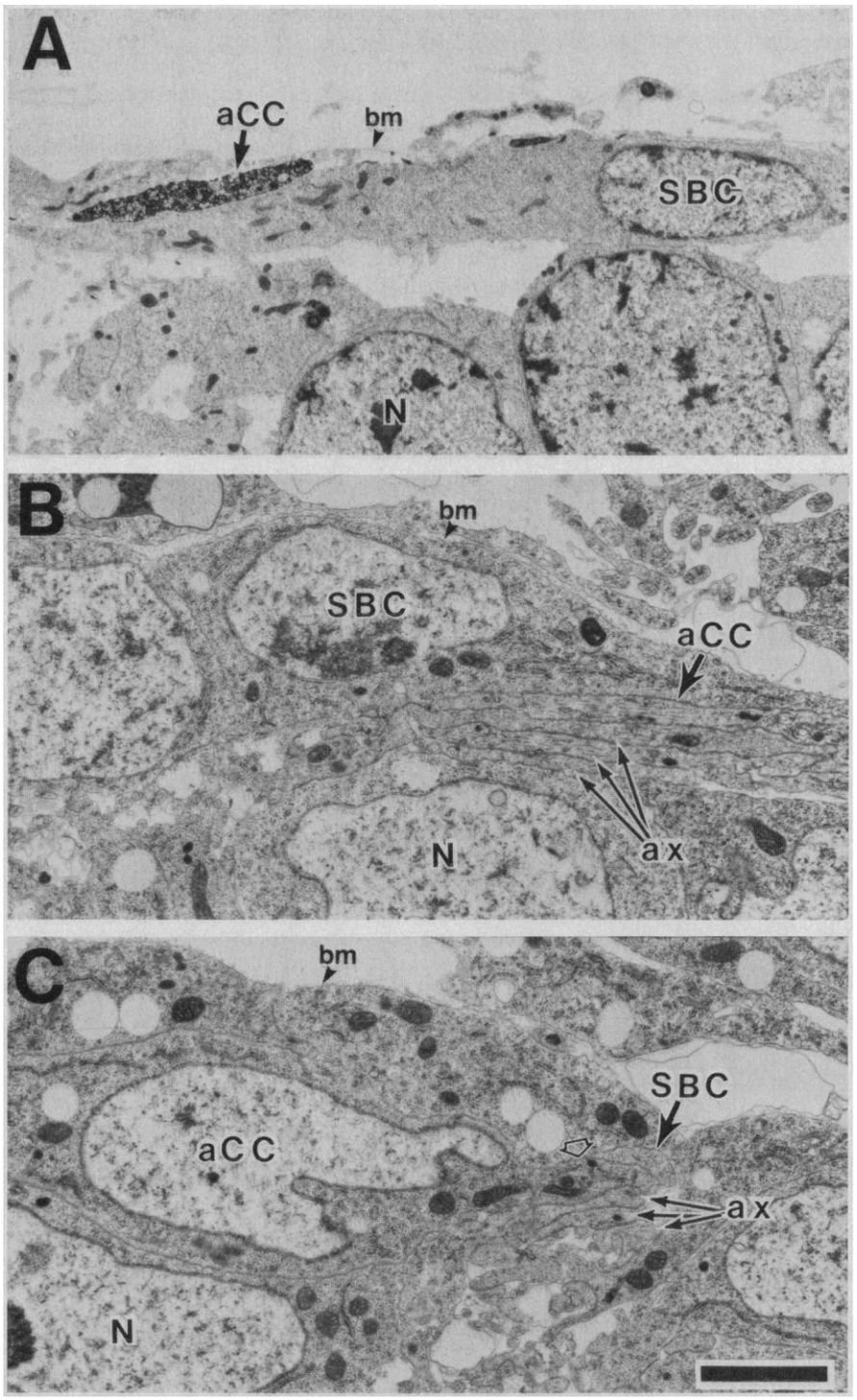

Fig. 4. Electron micrographs showing the relationship of the aCC neuron to the epidermal segment boundary cell (SBC) in the 35 percent grasshopper embryo (A) and the 10-hour *Drosophila* embryo (B and C). (A) In the grasshopper embryo, the aCC cell body is about 70 μm anterior to the segment border. The aCC initially waits for the U axons, which it then follows posteriorly to the segment border. At the segment boundary, it turns laterally along the SBC. (B) In the *Drosophila* embryo, the aCC cell body is less than 10 μm anterior to the segment boundary when it first extends its growth cone. Just as in the grasshopper, the aCC axon in the fly extends laterally at the segment boundary along the SBC. Here the aCC axon is shown along with three other axons (small arrows). (C) In the fly embryo, instead of waiting for the U axons, the aCC growth cone immediately extends posteriorly and then laterally along the SBC. The aCC neuron seems to be in contact with the SBC from the outset, since a process of the SBC extends anteriorly about 10 μm to the aCC cell body, and the aCC axon contacts and appears to run along this process (*30*). The U growth cones later fasciculate with the aCC axon, as do the growth cones of other identified neurons including RP1 and RP2 (Fig. 7B). Abbreviations: N, neuron; bm, basement membrane; ax, the other three axons extending out the intersegmental nerve at this stage. Scale bar: (A) 4 μm; (B and C) 1 μm.

ferently and are likely to express different surface labels. When one of these two progeny is ablated within 5 hours (1 percent) after their birth, the remaining cell differentiates into the pCC (Fig. 5E) (*28*). However, when ablations are made between 5 and 10 hours after their birth (just before they begin migrating), the remaining cell becomes either the aCC or the pCC with equal probability. The results suggest that the sibling progeny from the first GMC of NB 1-1 are (i) initially equivalent, (ii) become uniquely determined by early interactions, and (iii) exhibit a hierarchy of fates whereby the pCC is dominant (*28*).

In summary, the aCC and pCC neurons seem to be uniquely determined and to express different surface labels by the time they extend growth cones; they acquire these unique fates by a combination of their lineage and their early interactions.

The aCC and pCC Neurons in the *Drosophila* Embryo

Whereas the grasshopper embryo has been an ideal system for cellular studies of neuronal recognition, the *Drosophila* embryo has obvious attributes for a molecular genetic approach. The problem with studying neuronal recognition in the CNS of the *Drosophila* embryo has always been the small size of its seemingly inaccessible embryonic neurons. Until recently, nothing was known of the detailed cellular events underlying neurogenesis in the *Drosophila* embryo. However, in collaboration with Bate, we recently bridged this gap (*29*). By using scaled-down versions of the same cellular methods used to study the grasshopper embryo, we showed that the early *Drosophila* embryo CNS is a miniature replica of the grasshopper embryo in terms of its identified neurons, their growth cones, and their selective fasciculation choices (Fig. 6) (*29*). For example, the *Drosophila* embryo has a neuron homologous to the grasshopper G neuron. Just as in the grasshopper, the tip of the fly G growth cone associates with the P axons in the A-P fascicle.

Here we have described a simple model system in the grasshopper embryo: the cell recognition events mediated by the selective affinities of the aCC and pCC growth cones. These identified embryonic neurons extend their growth cones along the same stereotyped pathways in the fly embryo. The similarities between the development of these neurons in the grasshopper and *Drosophila* embryos are striking (*29*).

By hour 10, the first neurons have begun extending growth cones (Fig. 7A). As in the grasshopper, the growth cones of the MP1 and dMP2 extend posteriorly whereas that of the vMP2 extends anteri-

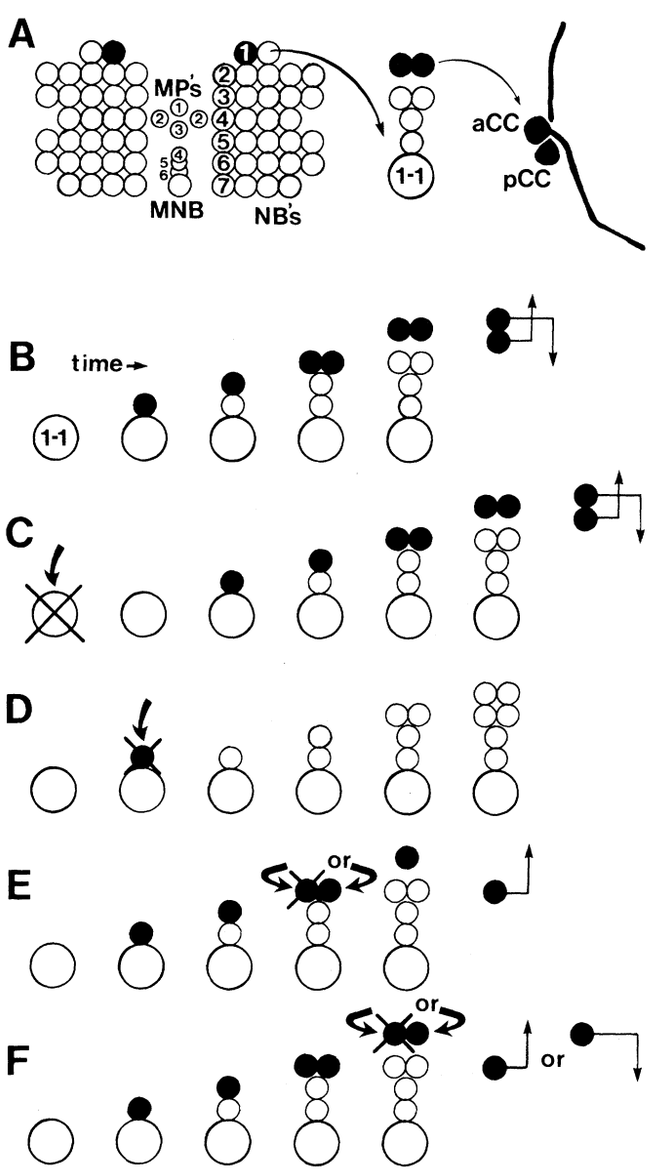

Fig. 5. Cell lineage and cell interactions generating the aCC and pCC neurons. (A) Pattern of neuronal precursor cells and cell lineage of aCC and pCC neurons from NB 1-1. Each segment contains a stereotyped pattern of two plates of 30 neuroblasts (NB's) (*24*), one median NB (MNB), and along the midline, seven midline precursor cells (MP's) (*17*). The NB's are stem cells that divide asymmetrically to generate ganglion mother cells (GMC's), which then divide symmetrically to give rise to pairs of neuronal progeny. The first GMC from NB 1-1 gives rise to the aCC and pCC neurons (*16*). In all parts of this figure, the first GMC or its neuronal progeny are filled circles. The aCC and pCC migrate anteriorly (shown by the separation of the black cells from the rest of the NB 1-1 progeny) and then differentiate into the two distinctive neurons. (B) Normal lineage of aCC and pCC neurons. (C) Ablation of NB 1-1. A new NB 1-1 appears and produces the aCC and pCC neurons (*27*). (D) Ablation of the first GMC from NB 1-1. The second GMC does not produce the aCC or pCC neurons (*27*). (E) Ablation of either of the progeny of the first GMC within 5 hours of their birth. The remaining cell differentiates into the pCC neuron (*28*). (F) Ablation of either of the progeny of the first GMC 5 to 10 hours after their birth: the remaining cell differentiates into either the aCC or pCC neurons with equal probability (*28*).

orly, pioneering the first two longitudinal axon fascicles. The pCC growth cone extends anteriorly as it fasciculates with the MP1 and dMP2 axons.

There is a temporal difference, however (*30*). In *Drosophila*, the aCC extends its growth cone posteriorly and then laterally to help form the intersegmental nerve, just as in the grasshopper. However, it does so at the same time as the extension of the MP1, dMP2, vMP2, and pCC growth cones (Fig. 7A), instead of 3 percent later as in the grasshopper (Figs. 2C and 3A).

This switch in timing between grasshopper and *Drosophila* seems to arise from the differences in distance from the aCC cell body to the large, conspicuous EC at the segment border, called the segment boundary cell (Fig. 4). The net result is that the aCC follows the U's in the grasshopper, whereas the U's follow the aCC in the fly. Despite the temporal switch in their order, the patterns of selective fasciculation displayed by these and other neurons are remarkably similar in grasshopper and *Drosophila*.

Monoclonal Antibodies as Molecular Probes for Cell Recognition Molecules

The next step in the analysis of neuronal recognition is to isolate potential recognition molecules and then test their function during neuronal development. Being able to work with identified neurons, their growth cones, and their patterns of selective fasciculation in the *Drosophila* embryo means that cellular, immunological, and molecular approaches can be combined with a genetic analysis to study the molecular basis of cell recognition during neuronal development. One approach is the use of monoclonal antibodies as probes for such recognition molecules in *Drosophila*.

Monoclonal antibodies (Mab's) generated against the grasshopper embryo CNS have revealed cell surface antigens expressed on small subsets of fasciculating axons (*31*). The temporal and spatial expression of these surface antigens during embryogenesis is related to our prediction that neurons whose axons fasciculate together share common surface antigens (*31*). Mab's have also been generated against fasciculating axons in the leech nervous system (*32*). In the grasshopper embryo, for example, two Mab's (Mes-3 and Mes-4) recognize surface antigens that label the MP1-dMP2 fascicle and distinguish it from all of the other approximately 25 longitudinal fascicles in the 40 percent embryo (*31*). One other Mab (Mes-2) recognizes an antigen transiently expressed on the surface of only four of 1000 neurons in each hemisegment, two of whose axons selectively

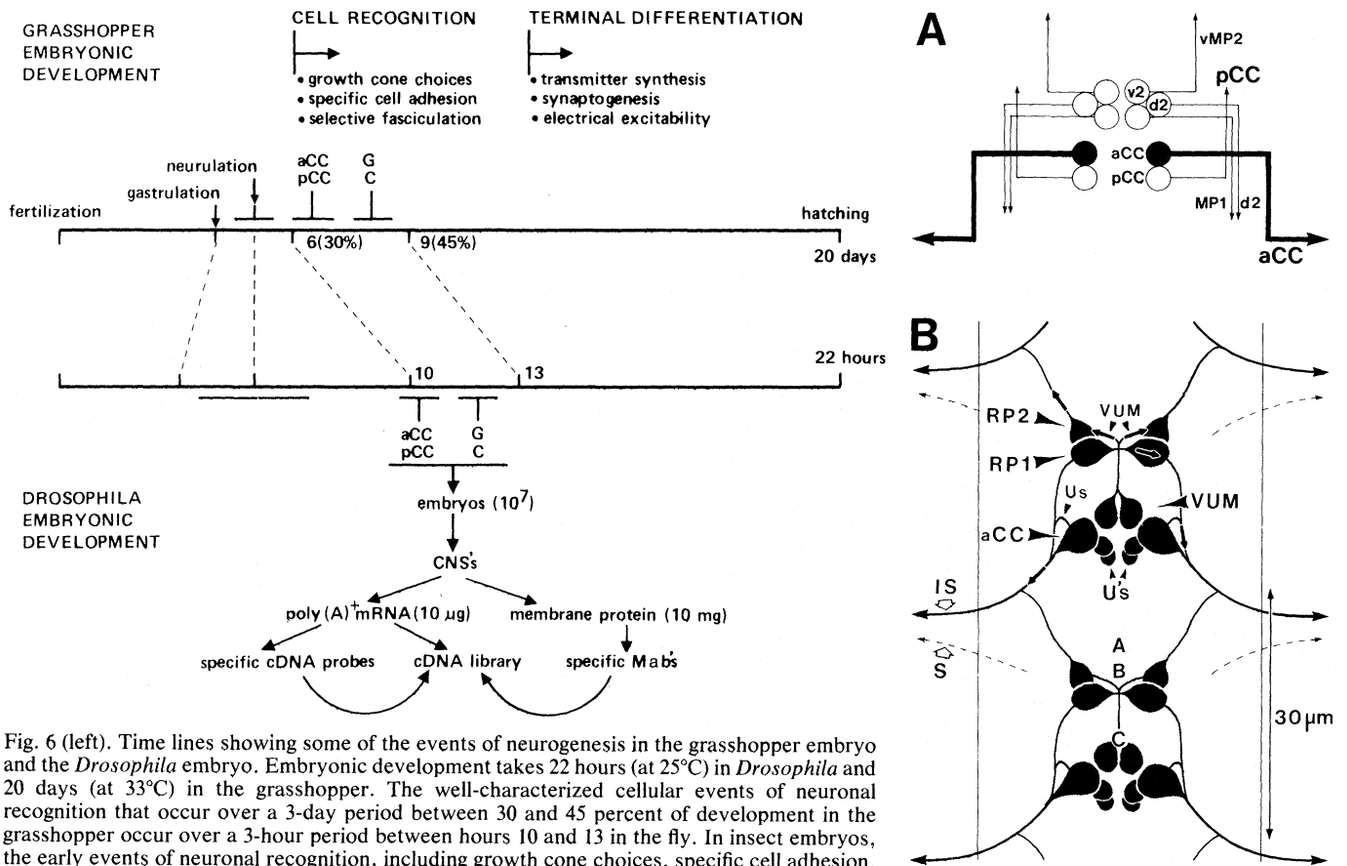

Fig. 6 (left). Time lines showing some of the events of neurogenesis in the grasshopper embryo and the *Drosophila* embryo. Embryonic development takes 22 hours (at 25°C) in *Drosophila* and 20 days (at 33°C) in the grasshopper. The well-characterized cellular events of neuronal recognition that occur over a 3-day period between 30 and 45 percent of development in the grasshopper occur over a 3-hour period between hours 10 and 13 in the fly. In insect embryos, the early events of neuronal recognition, including growth cone choices, specific cell adhesion, and selective fasciculation, occur before the appearance of neurotransmitters, synapses, and electrical excitability (*25, 43*). Neurulation refers to the period during which the NB's (the major class of neuronal precursor cells) are appearing. Fig. 7 (right). Selective fasciculation and expression of the SOX2 antigen in the *Drosophila* embryo. (A) Schematic diagram of a 10-hour *Drosophila* embryo showing the selective fasciculation by the growth cones of the aCC, pCC, MP1, dMP2, and vMP2 neurons. The aCC extends posteriorly and then laterally at the segment border along the SBC (not shown; see Fig. 4, B and C). Of these five identified neurons in each hemisegment at this stage, only the aCC expresses the SOX2 antigen on its surface (denoted by black cell body and axon). (B) Schematic camera lucida drawing of some of the identified neurons expressing the SOX2 antigen at about 12 hours of embryonic development in *Drosophila* (*39*), as drawn in two segments. Vertical line on right denotes a single segment; arrowheads mark segment borders. The SOX2 antigen is expressed on the surface of the aCC and other neurons whose axons fasciculate in the intersegmental nerve (IS). The ventral unpaired median (VUM) neurons are homologous to grasshopper DUM neurons; several other motoneurons, whose cell bodies are ventrolateral and whose axons fasciculate in the intersegmental nerve, express the SOX2 antigen as well but are not drawn because we do not yet know their complete axonal morphology. The shaded area shows the extent of the embryonic neuropil containing the orthogonal scaffold of axon fascicles; the dashed line shows the segmental nerve (S).

fasciculate together while they express the antigen (*31*).

The next step is to isolate and characterize the molecules and understand how they function. One way to isolate the genes encoding these potential cell recognition molecules is to use Mab's in conjunction with either expression cloning (*33*) or microsequencing and oligonucleotide probes (*34*). Function can then be tested by genetic analysis in *Drosophila*. Unfortunately, none of the Mab's against the grasshopper cross-react with similar neuronal surfaces in the *Drosophila* embryo. Thus, we generated new Mab's against the *Drosophila* CNS from 10- to 13-hour embryos. Starting with a collection of 10 grams (10^7 embryos) of 10- to 13-hour embryos, we use a "mash" technique (*35*) to isolate more than 10^5 10- to 13-hour CNS's (80 to 90 percent pure). Such CNS preparations generate 10 mg of crude membrane protein for the immunization of mice (*36*), and 10 μg of poly $(A)^+$ messenger RNA for the construction of a 10- to 13-hour CNS-specific complementary DNA library for expression cloning (*37*).

Between 10 and 13 hours of development, the CNS of the fly embryo consists largely of 14 repeated segmental units. Each hemisegment contains a stereotyped pattern of about 100 different neurons. Thus, each isolated nervous system contains 28 repeats of a basic 100-neuron unit. Using crude membrane preparations of this material to immunize mice and suppression methods designed to reduce the response to ubiquitous surface antigens (*38*), we have generated several Mab's that recognize surface antigens expressed on subsets of fasciculating axons during embryonic development (*39*).

Monoclonal Antibody Distinguishes the Surface of the aCC from the pCC Neuron

Hybridomas are screened on isolated whole mount CNS's from 10- to 13-hour-old fly embryos. Screening 1000 clones from four fusions, we have isolated four Mab's that recognize surface antigens expressed on subsets of embryonic axons early in *Drosophila* development (*39*). Here we briefly describe the expression of the SOX2 antigen (Figs. 7B and 8, B and C) on a subset of neurons in the CNS whose axons fasciculate as they extend out the intersegmental nerve (Figs. 6B and 8B) and on peripheral sensory neurons whose axons fasciculate as they extend into the CNS along the intersegmental and segmental nerves (Fig. 8C).

At hour 10, the SOX2 antigen is expressed on the surface of the aCC in every hemisegment, but not on the pCC, MP1, dMP2, or vMP2 neurons (Fig. 7A). By hour 11, several additional identified neurons express the SOX2 antigen (Fig. 7B). In total, about 5 percent of the neurons in the CNS express the SOX2 antigen, and their axons selectively fasciculate during embryogenesis.

The expression of the SOX2 antigen on a small subset of neurons whose axons show a high affinity for one another during development suggests that this molecule may play a role in neuronal recognition. Moreover, the SOX2 Mab distinguishes between the aCC and pCC neurons, in accord with our cellular analysis that predicted that these two sibling neurons express different surface labels. The SOX2 antigen is thus a prime candidate for further molecular genetic studies.

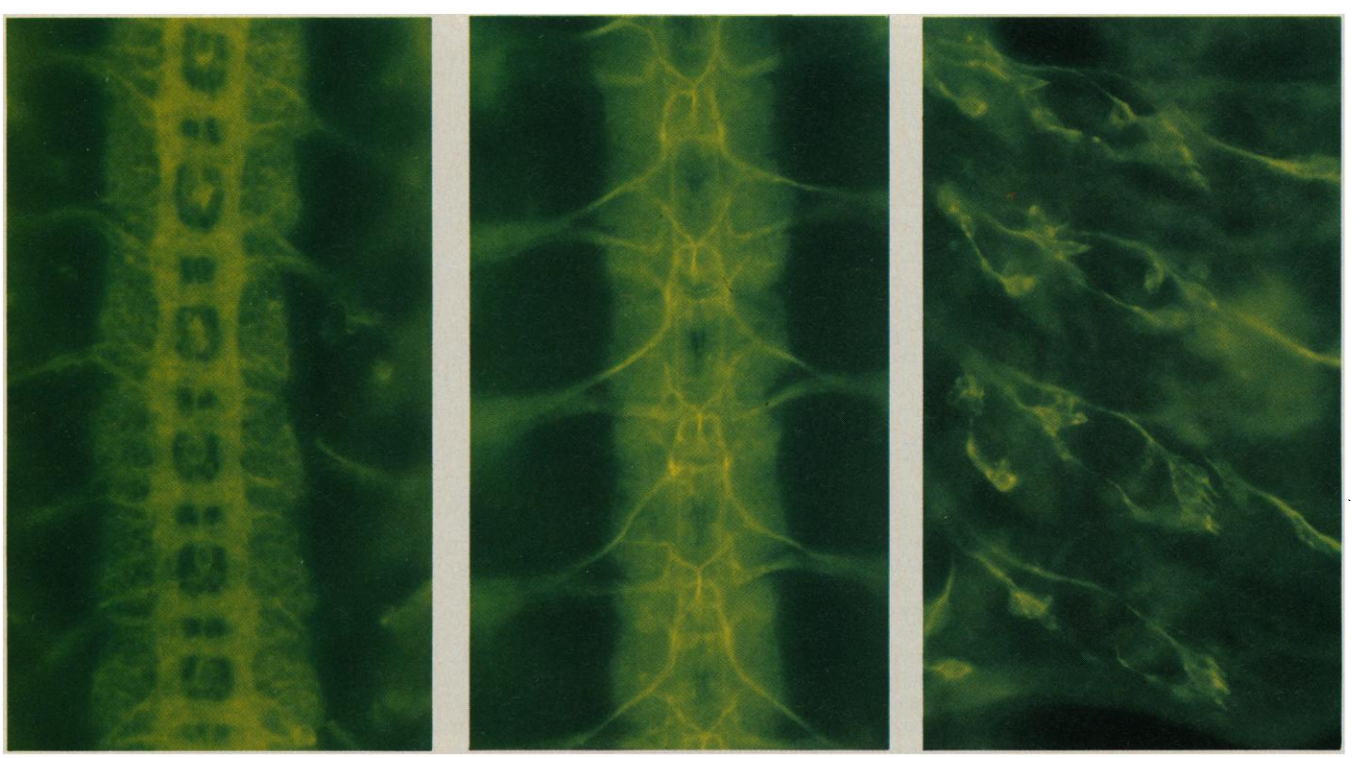

Fig. 8. The SOX2 Mab stains a surface antigen expressed on a subset of embryonic neurons in the *Drosophila* CNS (*39*). (A) Staining of the neuropil by an antibody to tubulin; (B) staining of a subset of neurons in the CNS; and (C) peripheral sensory neurons by the SOX2 Mab at about hour 12. [The embryo shown in (A) may be about 15 minutes older than that shown in (B) and (C).] Photomicrographs of whole mount CNS's showing either five (A) or four (B and C) embryonic segments in the CNS (A and B) or peripheral tissues (C). The SOX2 antigen is expressed on the aCC and other neurons whose axons fasciculate with the aCC as they extend out of the CNS in the intersegmental nerve (IS) (see Fig. 7B). The SOX2 antigen is also expressed on most if not all peripheral sensory neurons whose axons extend into the CNS in both the intersegmental and segmental nerves. By hour 12, one medial longitudinal axon fascicle begins staining with the SOX2 Mab. As of yet we have been unable to identify any central neurons whose axons run in this fascicle, but rather have preliminary evidence that it contains the axons of some of the peripheral sensory neurons. In (B), each segment is 30 μm long (see Fig. 7B for identification of neurons and nerves).

Future Prospects

The cellular analysis of neuronal recognition in both the grasshopper and *Drosophila* embryos indicates that surface recognition molecules are expressed on subsets of neurons early in development. Monoclonal antibodies reveal surface antigens whose expression correlate with this prediction. The next step is to use these Mab's as molecular probes to isolate the genes encoding these molecules and test their function.

A detailed understanding of the molecular basis of cell recognition during neuronal development may be within reach in the *Drosophila* embryo. The advent of recombinant DNA and Mab techniques, the advances in gene transformation methodologies (*40*), the genetics of *Drosophila*, and the new ability to examine its developing nervous system in great cellular detail (*29*) make this an ideal experimental organism. Most important is that the function of these molecules can be examined in ablation and transplantation experiments through the use of genetic mutations and deletions, gene fusion, and gene transformation techniques.

Many exciting questions remain for future studies. For example, what is the molecular code specifying cell recognition in the nervous system? How many different molecules are used, and are they members of multigene families? To what extent is the molecular specificity encoded by different proteins or by different glycosylations of the same protein? Are these specific recognition molecules in fact specific cell adhesion molecules (*41*)? What controls the expression of these recognition molecules? To what extent is their expression a dynamic process that changes during development? Finally, are the same or related molecules used to specify both the early events of selective fasciculation and the later events of synapse formation? Over the next decade, we may hope to glimpse how the simple nervous system of insects becomes properly wired during development.

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36. Nervous systems were isolated by the "mash" technique (*35*), sonicated and then centrifuged at 4°C, and the pellet resuspended in phosphate-buffered saline for intraperitoneal or intravenous injections (or both).
37. Starting with approximately 10 μg of poly $(A)^+$ messenger RNA, a complementary DNA library was constructed from this 10- to 13-hour CNS messenger RNA in the bacteriophage lambda vector, gt10 (P. Patten, unpublished results). For expression cloning, this library has been converted to lambda gt11 (*33*) (S. Crews, unpublished results).
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