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Surface Molecules Identify Groups of Growing Axons

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I. Thompson, K. Frederiksen

Summary. Studies on vertebrate and invertebrate species have established that, during development, axons have the ability to choose particular paths over others. The chemical basis of this pathfinding is not clear but biochemical differences between neurons have long been postulated to account for the specificity of neuronal connections. Such subtle molecular differences between different cells in a single tissue are difficult to study with standard biochemical techniques but hybridoma technology has offered a potential solution to this type of problem. This technique has made possible the production of monoclonal antibodies for identifying and characterizing a family of glycoproteins which are expressed on the surface of specific axon bundles during the development of the leech nervous system. The results show that groups of growing axons do indeed carry chemically distinct surface molecules.

In contrast to those of vertebrates the nervous systems of many invertebrates contain relatively few neurons. In the leech, for example, the central nervous system is made up of a total of 10^4 neurons, but these are arranged in repeating similar ganglia, each composed of only 400 neurons. Within a ganglion many of the neurons are bilaterally symmetrical so the basic unit of the leech nervous system is a 200 cell half ganglion. This anatomical simplicity of the organization of neuronal cell bodies in the leech offers a great advantage as an experimental system (*1*). We made use of this anatomical simplicity to screen a large number of monoclonal antibodies, raised against the dissected nerve cord, for the ability to recognize antigens present in subsets of neurons (*2*). The initial fusions generated several hundred hybridoma cell lines.

Using a whole mount preparation of the leech ganglion as our immunohistochemical assay, we found that approximately 10 percent of these lines secreted antibody that bound to subsets of neurons. These monoclonal antibodies, which bound to subsets of neurons, often bound to overlapping subsets; but in no cases in our initial studies did we find two antibodies which recognized the same subset of neurons. As we saw more than 30 different staining patterns without a repeat, there is a high statistical probability that there are more than 200 differentially distributed antigens in the leech nerve cord. These data suggest that the nervous system is made up of a complex series of chemically differentiated cell types; they are consistent with measurements of a high degree of neuronal complexity obtained by different methods in vertebrate nervous systems (*3, 4*). These data are also consistent with models where synaptic networks and other differentiated physiological functions and anatomical features of the nervous system result from molecular differences between neurons.

Another important consequence of the production of antibodies to antigens in subsets of neurons is that these antibodies have allowed us to see surprising new features in the cellular organization of the leech nervous system and that of vertebrate (*5–7*). In the leech, we have made use of the simple anatomy of its central nerve cord to show that axons are organized into stereotyped groups (*5*). The leech central nervous system consists of a chain of segmental ganglia. Large bundles of axons, the connectives,

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run between each pair of ganglia, allowing for the integration of neuronal signals and behavior over the length of the animal. Each connective contains approximately 5000 axons that form two large lateral bundles and one smaller medial bundle. These bundles are subdivided into smaller groups, axon fascicles, by the process of two glial cells. Some of the axons in the connective arise from ganglionic neurons, others arise from peripheral neurons. We have shown with our panel of monoclonal antibodies that the positions of antibody-identified subsets of axons in the leech connective occupy highly stereotyped positions and are often grouped into axon fascicles (*5*). These results and the results of experiments in which we have located the axons of physiologically identified neurons (*8*) suggest that functionally related neurons can be identified by the location of their central axons. The developmental events that place axons next to particular neighbors is likely to be an important part of the mechanism that establishes specific synaptic relations between neurons.

Because of the possible importance of the observation that axons maintain precise anatomical relations as they travel through the connective, we have extended our analysis of the organization of axons. In this article we describe the use of two monoclonal antibodies to obtain a more detailed understanding of the anatomy of adult and embryonic axons. We also show that the antigenic differences associated with the surface of subsets of axons in the leech connective are carried on glycosylated, protease sensitive molecules. Developmental studies show that this family of protease sensitive, glycosylated antigens are differentially expressed on some axons from early stages of axon outgrowth.

Surface Antigens on Axon Subsets

Each of the two monoclonal antibodies we have used in this study, Lan 3-2 and Lan 4-2, recognize a subset of mechanosensory neurons. Each standard segmental ganglion of the leech nerve cord contains seven pairs of large, bilaterally symmetrical, mechanosensory neurons. Included in these are two pairs (one medial and one lateral pair) of neurons that respond to noxious stimulation of the skin, the N cells (*9*). Monoclonal antibody Lan 3-2 recognizes both the medial and lateral pair of N cells; antibody Lan 4-2 recognizes only the lateral pair in each standard segmental ganglion (Fig. 1). In addition to staining cell bodies, Lan 3-2 and Lan 4-2 stain symmetrically disposed groups of axons in the ventrolateral quadrants of the connective (Fig. 2, A and D). The positions of these stained axons is consistent over the rostrocaudal axis of each animal and among animals of the same species. Both antibodies stain subsets of the axons in the lateral axon bundles.

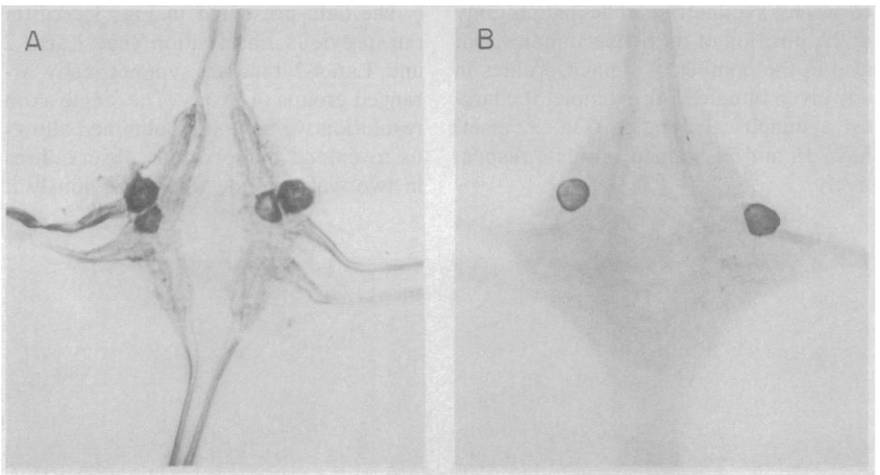


Fig. 1. Antibody binding to subsets of cell bodies. Lan 3-2 binds to all four nociceptive cell bodies (A) and Lan 4-2 binds only to the lateral nociceptive cell bodies (B) of *Hemopis marmorata* midbody ganglia (*21*). The derivation of the monoclonal antibodies Lan 3-2 and Lan 4-2 has been described previously (*2*, *5*, *9*). Immunohistochemistry at the light microscope level. Fixed whole mounts of adult leech ganglia were processed as described previously (*2*). The immunohistochemical analysis of the developing nervous system was performed as in the adult; the dissection of 4 percent formaldehyde fixed leech embryos was carried out by fine tungsten mounted wires.

Electron microscopy of connectives stained with each of these antibodies shows that antibody-positive axons travel together in fascicles. Most antibody-positive bundles of axons are bound by the processes of the connective glial cells. Figure 2C illustrates a fascicle of Lan 3-2 positive axons delimited by glial cell processes and surrounded by fascicles of antibody-negative axons. While the staining patterns of Lan 3-2 and Lan 4-2 are similar by light microscopy, electron microscopy shows that Lan 4-2 and Lan 3-2 recognize overlapping sets of axons; Lan 3-2 predominantly stains small-diameter axons (0.1 to 0.5 μm), and Lan 4-2 stains both small and large diameter (greater than 0.5 μm) axons (Fig. 2, C and F).

Lan 3-2 and Lan 4-2 bind to surface antigens by two criteria. First, in high magnification electron micrographs of axons (Fig. 2, C and F) antibody staining is associated with the perimeter of stained axons while the axoplasm is free of stain. Second, Lan 3-2 stains the four N cells in unfixed, living preparations of leech ganglia where the connective tissue capsule of the ganglion has been opened to allow the monoclonal antibody access to the surface of neuronal cell bodies. When these preparations were fixed after antibody staining and processed for electron microscopy, the antigen was clearly present on the outer surface of labeled neurons (*9*).

Symmetrical Organization of Axon Fascicles

The symmetrical organization of antibody-labeled axons is shown in the light micrographs in Fig. 2, A and D. Further analyses by electron microscopy revealed the features of axon symmetry in greater detail. The symmetry in the organization of antigenically identified bundles of axons (Fig. 3) was diagramed by tracing antibody-identified bundles of axons in a montage of low magnification electron micrographs from a single cross section of the connective. Serial sections adjacent to the one illustrated were examined to ensure that antibody penetration was uniform. This confirmed that the selective staining of axons was not a result of uneven antibody accessibility. Using a new technique which improved antibody penetration (*10*), we could accurately compare the positions and organization of bundles of axons on both sides of the connective and increase the resolution of stained profiles to the level of single axons. High magnification electron micrographs of corresponding symmetrical bundles of axons from the diagramed cross section of the connective show more precisely the organization of stained axons (Fig. 3, inset). These micrographs show that while the symmetry of axon organization is clear, it is not geometrically perfect. The lack

of perfect symmetry is reflected not only in the position of particular bundles, but also in the number of stained profiles in any given bundle. For example, the largest symmetrical bundles (Fig. 3, inset) have 16 and 21 stained profiles, respectively.

The data presented in Fig. 3 confirm our previous observation that Lan 3-2 and Lan 4-2 bind to symmetrically arranged groups of axons. The single axon resolution we have now obtained allows us to extend our previous observations in two ways. First, where previously it was unclear whether the antigen was present on all the axons in a bundle or only on the most peripheral axons, we now have shown that all the axons throughout many fascicles share a surface antigenic determinant. Second, because we can now count the number of axons recognized by an antibody we have shown that while the symmetrical organization of axons is clear the precise features of fasciculation are not perfectly conserved.

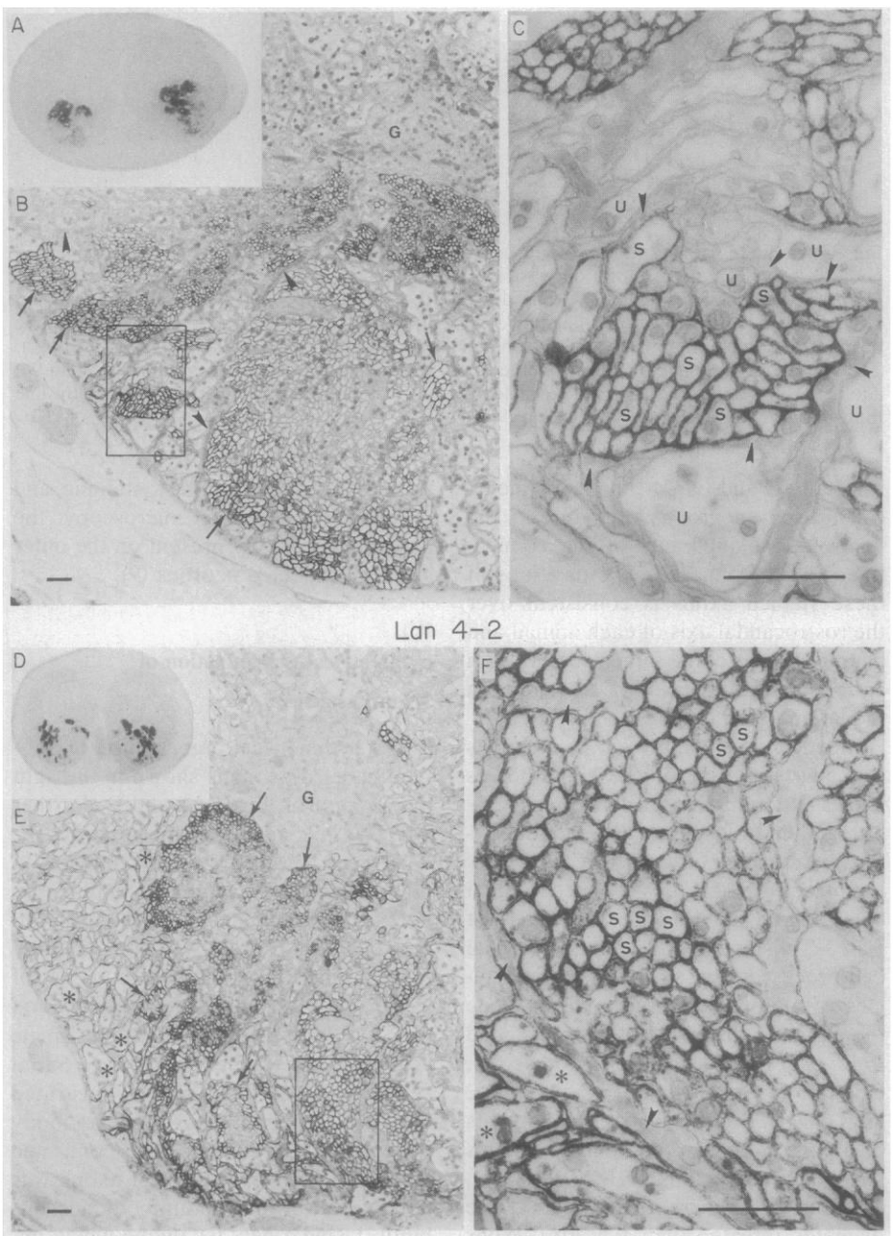


Fig. 2. Antibody binding to subsets of axons. Cross sections of the connective shown at the light level (A and D), at low power (B and E) and at high power (C and F) electron microscopy. The symmetrical organization of axon subsets is shown in the light micrographs. More details of the organization of antibody identified axon fascicles can be seen in the electron micrographs. The glial cell is marked *G* and the processes of the glial cell are indicated by arrowheads. Fascicles of labeled axons are marked by arrows. Stained and unstained axon profiles are marked *S* and *U* respectively. Lan 4-2 recognizes a set of larger axons; examples of these are marked with an asterisk. Following the protocol of Eldred *et al.* (*10*), adult nerve cord or whole leech embryos were fixed in 4 percent paraformaldehyde at *p*H 7.4 (30 minutes, room temperature) followed by 4 percent paraformaldehyde at *p*H 1.0 (2 to 12 hours, 4°C). Small pieces of tissue were immersed in 1 percent sodium borohydride (30 minutes), washed in buffer and then run through graded alcohols (10, 20, 40, 20, and 10 percent) back to buffer. Tissue was incubated in first antibody overnight, washed in buffer and second antibody [horseradish peroxidase conjugated rabbit antibody to mouse antiserum (Cappel)] for 2 hours. Peroxidase was visualized with 3,3′-diaminobenzidine; the tissue was postfixed in 2 percent OsO_4 and embedded in Epon-Araldite. Sections from the first 5 to 10 μm of the tissue surface were examined unstained in the electron microscope.

Embryological Distribution of Lan 3-2 Antigen

We have examined the developmental appearance of the Lan 3-2 antigen in two species of leech, *Hemopis marmorata* and *Helobdella triserialis*. The central nervous system (CNS) of *Helobdella* does not contain Lan 3-2–positive cell bodies but does contain the symmetrically disposed groups of Lan 3-2–positive axons which are also stained in *Hemopis marmorata* (*5*). In early embryos of both these species Lan 3-2–positive axons and cell bodies are first seen in the peripheral skin. Axons carrying these specific antigens grow from the cell bodies in the periphery into the CNS.

The development of Lan 3-2–positive axons in *Helobdella triserialis* is shown in Fig. 4. The antigen is first seen in groups of peripheral cells aligned along the central annulus of each segment on the dorsal body wall. In Fig. 4A, a section of the body wall of an early stage 11 embryo is shown stained in whole mount; several groups of peripheral neurons already express the Lan 3-2 antigen in their cell bodies and processes. Each more central group of cells (marked *C*) is progressively less differentiated, but the cell bodies and processes are already antigenically positive. At high magnification (Fig. 4B) the cell bodies and processes of one of the group of cells marked *P* in Fig. 4A. We can see that, in addition to the major processes, a network of fine antigenically positive processes are present. These fine processes, which are close to the limit of resolution of the light microscope, are detectable because of the deposition of horseradish peroxidase reaction product. The size and morphology of these fine processes suggest that they are the filopodia of the growing processes of axons.

The processes of the peripheral cell groups elongate toward the CNS forming distinct fiber bundles with the processes of more centrally located groups of neurons. When these processes reach the CNS they arborize within the ganglia

(Fig. 4D). Here again, immunocytochemistry shows by light microscopy that the arbors formed by these peripheral neurons carry the Lan 3-2 antigens (Fig. 4C). As the processes grow further in the CNS, they send branches rostrally and caudally to form symmetrically arranged rows of axons in the midbody ganglia (marked *G* in Fig. 4D). Figure 4D shows the relation between the peripheral neurons and the antigenically positive central axons. As we are able to stain transient structures, filopodia, and central arbors, we are able to conclude that the antigens are part of the growing tip of the axon.

The disposition of Lan 3-2–positive axons in the isolated CNS of the late stage 11 embryo is shown in Fig. 4E. In this case, the CNS was dissected and stained in whole mount. In the rostral body segments, a well-developed fiber pattern is apparent; in more caudal segments the fiber tracts are less well developed. This observation is consistent with the general rule of a rostral-caudal gradient of differentiation. An exception is seen in the most caudal seven ganglia which later form the tail ganglion. In the tail ganglia, the Lan 3-2–positive fibers grow into the ganglia and arborize extensively at early stage 11, a time when the head ganglia are the only other antigenically positive central structures. In spite of their early appearance in the tail ganglia, antigenically positive fibers do not cross into the most caudal of the midbody ganglia even though these ganglia are directly adjacent to one another.

In embryonic as well as adult *Helobdella*, Lan 3-2–positive cell bodies have only been observed in the periphery; the adult central nervous system contains Lan 3-2–positive axons but not cell bodies. Our embryological studies indicate that many of these central axons are derived from peripheral neurons (Fig. 4). The distribution of axons in the adult nerve cords of *Hemopis marmorata* and *Hirudo medicinalis* is similar to that seen in *Helobdella*. We have obtained *Hemopis* embyros from leeches captured from the wild. The general pattern of development is similar to that seen in *Helobdella*; that is, segmentally arranged rows of peripheral groups of Lan 3-2–positive cell bodies send their processes into the CNS. These fibers arborize to give symmetrically disposed groups of axons. This is shown in Fig. 5A where symmetrically arranged axons travel through a ganglion (the neuronal cell bodies are marked *N*). Immunoelectron microscopy shows that in the connective and neuropil of the *Hemopis* embryo the Lan 3-2 antigen is present on the surface of developing processes (Fig. 5B). These antigenically positive processes run in bundles before the processes of glial cells have separated them from unstained neighboring axons. Similarly, electron microscopy of *Helobdella* embryos shows that the Lan 3-2 antigen is present on the surface of axons that form fascicles early in development.

Our developmental data shows that Lan 3-2 recognizes surface antigens expressed by neurons from the earliest stages of axon outgrowth and that antigenically positive bundles of axons occupy symmetrical locations in the connective at early stages in development (Figs. 4 and 5).

Biochemical Characterization of Lan 3-2 and Lan 4-2 Antigens

One of the technical advantages in using monoclonal antibodies to study complex tissues is that the same reagents can be used for both immunohistochemical and biochemical analysis. Our histochemical studies described here show that the antigens recognized by Lan 3-2 and Lan 4-2 have an anatomical distribution in the adult and embryonic nervous system which shows that the surfaces of different groups of growing axons can be chemically distinct. Here we describe experiments designed to determine the biochemical nature of the molecules carrying these surface antigenic determinants.

On immunoblots of proteins extracted from the leech central nervous system, Lan 3-2 binds to a series of high molecular weight antigens. Figure 6 shows that Lan 3-2, in contrast to other immunoglobulin Gl (IgGl) monoclonal antibodies, specifically recognizes three major bands between 90,000 and 130,000 daltons in the species *Hemopis marmorata*. All three of these bands are sensitive to treatment with protease K (Fig. 6, lane 4).

The anatomical data suggest the possibility that Lan 3-2 and Lan 4-2 recognize related antigens. This possibility is strengthened by the biochemical results shown in Fig. 6B. Proteins extracted from the nerve cord of *Hemopis mar-*

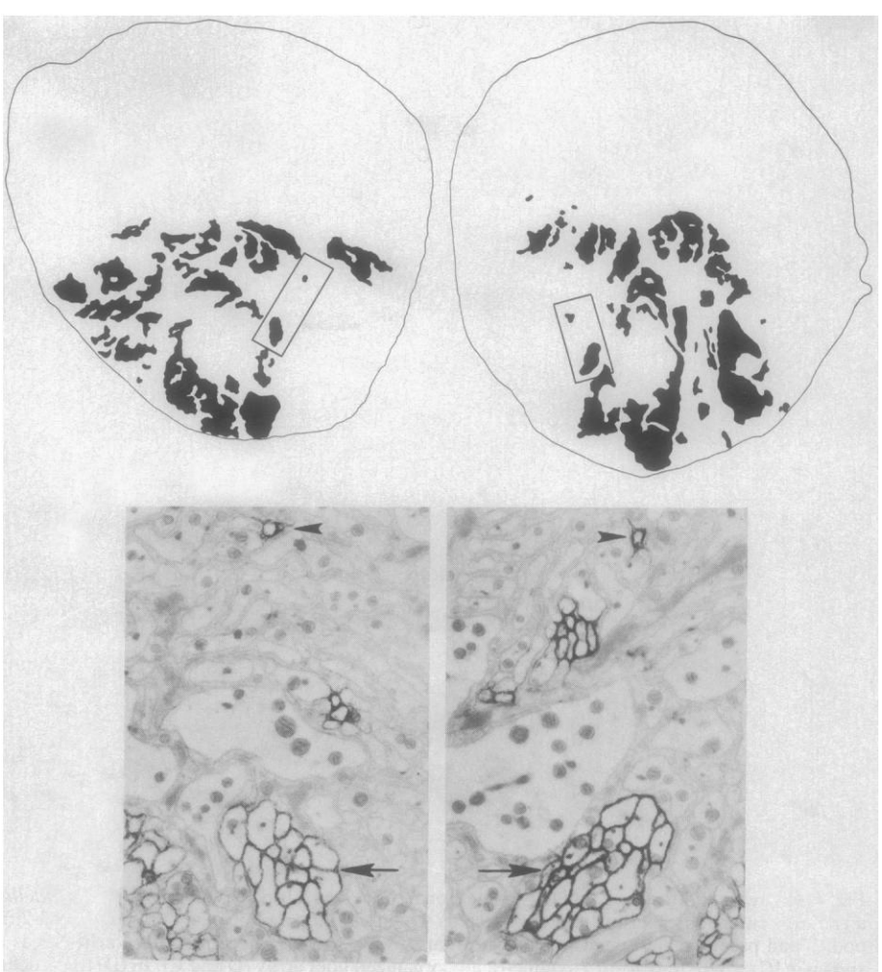


Fig. 3. Diagram showing the distribution Lan 3-2 identified axon bundles. The boxes marked in the diagram are shown in the inset electron micrographs.

morata were analyzed with the immunoblot procedure (Lan 4-2, Fig. 6B, lane 1; Lan 3-2, Fig. 6B, lane 2). Lan 4-2 binds strongly to a 130,000-dalton band which comigrates with the band of antigens of high molecular size recognized by Lan 3-2. Like the antigens recognized by Lan 3-2, the Lan 4-2 130,000-dalton antigen is protease sensitive.

The Lan 3-2–positive antigens can be extracted from the leech central nervous system in high yield by 1 percent Triton X-100. The solubilized antigens were selectively bound by lectins covalently linked to agarose beads. The Lan 3-2 antigens from *Hirudo medicinalis* bind to concanavalin A (Con A) beads and lentil lectin beads but not to beads carrying wheat germ agglutinin (WGA) (Fig. 7). These antigens can be specifically eluted with sugar and no additional antigen is subsequently eluted by sodium dodecyl sulfate and mercaptoethanol. Lectin binding experiments also show that the antigen bound by Lan 4-2 is glycosylated. It will be interesting to determine in more detail the chemical relation between these antigenically reactive bands. Our biochemical data are consistent with our immunohistochemical observations, as we might expect to find glycosylated proteins on the surface of cells. Since these antigens can be easily extracted from leech nerve cord and are glycosylated and protease sensitive, we suggest that they form a family of related surface glycoproteins. Whether there are additional members of this family of antigens present on the surface of different axon subsets remains to be determined.

The fact that the Lan 3-2 antigens are glycosylated raises the possibility that the antigenic determinant recognized by Lan 3-2 is itself a carbohydrate structure. Accordingly, nitrocellulose strips carrying the leech antigens were incubated with increasing dilutions of Lan 3-2 in the presence and absence of various sugars. The binding of Lan 3-2 to the nitrocellulose bound antigen was specifically inhibited by methylmannoside and not by other sugars (Fig. 8). This observation suggests that the antigenic determinant recognized by Lan 3-2 is composed, at least in part, of carbohydrate. The antigens recognized by Lan 4-2 are also glycosylated since they can be specifically bound to and eluted from lectin beads, but no sugar we have tested blocks the binding of Lan 4-2 in this assay (*9*).

Significance of Stereotyped Axon Organization

We show here that two antibodies against surface determinants bind to physiologically related cell bodies, anatomically related axons and similar protease sensitive, glycosylated antigens. These antigens are expressed from early stages of neurite outgrowth. Therefore during process outgrowth, axon surfaces can carry specific surface markers (glycosylated proteins) that distinguish the

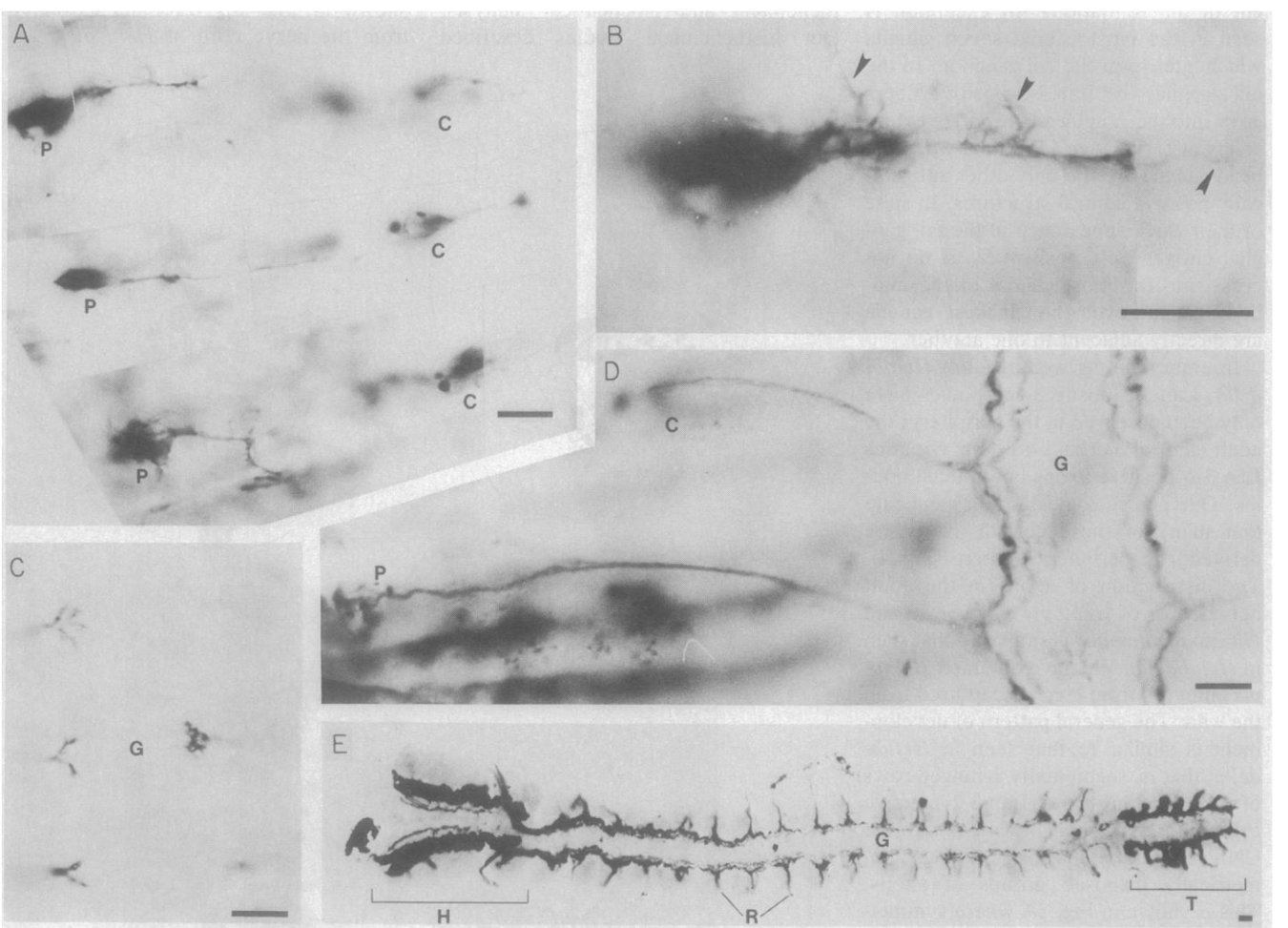
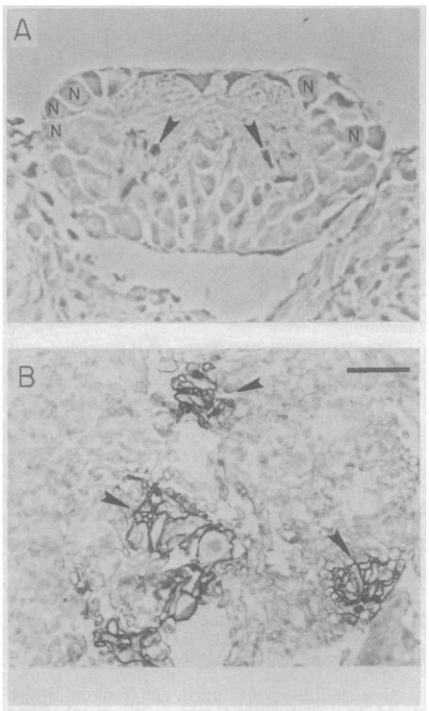


Fig. 4. Expression of Lan 3-2 antigens on neuronal processes during development in *Helobdella triserialis*. In the middle annulus of each segment a group of cutaneous neurons express antigens which bind the monoclonal antibody Lan 3-2 from early stages of neurite growth (A). These cell bodies and processes at higher magnification are shown in (B). These processes arborize as they grow into the central ganglia (marked *G*) as shown in (C) where they form a symmetrically organized fiber array (D and E). In (D) the processes extending from the cutaneous cells (marked *P* and *C*) can be seen extending into the central ganglia. In (E), a stage 11 nervous system is shown in whole mount after staining with Lan 3-2. All the preparations shown here were obtained from stage 11 embryos (*22*). All scale bars represent 10 μm.



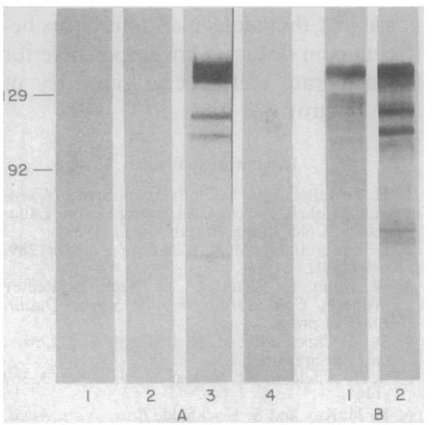


Fig. 5 (left). Symmetrically organized antigenically distinct axon fascicles in the developing cord of *Hemopis marmorata*. By light microscopy, symmetrically organized groups of antigenically positive processes can be seen in this cross section through a ganglion. Neuronal cell bodies are marked *N* and labeled processes indicated by arrowheads. By electron microscopy, these processes are seen to carry antigen on their surface and run in bundles (B). The scale bar represents 1.75 μm. Fig. 6 (right). (A) Lan 3-2 binds to specific antigens on Western blots. Leech neural antigens were extracted from the dissected nerve cord by homogenization in Laemmli loading buffer and boiling. The solubilized material was placed on polyacrylamide gels (10 percent) and electro-transferred to nitrocellulose. The lanes carrying material from five ganglia were challenged with control monoclonal antibodies of the same subclass as Lan 3-2 in lanes 1 and 2 and Lan 3-2 in lane 3. The bound monoclonal antibodies were visualized with second mouse antibody conjugated to peroxidase. Exposing the solubilized antigen to protease K before gel electrophoresis abolished antibody binding (lane 4). (B) Lan 4-2 binds to a major high molecular weight antigen (lane 1) which comigrates with the Lan 3-2 high molecular weight antigen (lane 2). Leech antigens were extracted from the nerve cord in Laemmli buffer (*23*). After electrophoresis on 10 percent polyacrylamide gels, the antigens were electroblotted onto nitrocellulose paper and challenged with antibodies (*24*). Peroxidase conjugated second antibody (1:200, Miles-Yeda) was used as a tracer and chloronaphthol was used as a substrate. In lane 4, the sodium dodecyl sulfate and mercaptoethanol-solubilized leech antigens were treated with 5 μg of protease K (Boehringer-Mannheim) per milliliter for 20 minutes before electrophoresis.

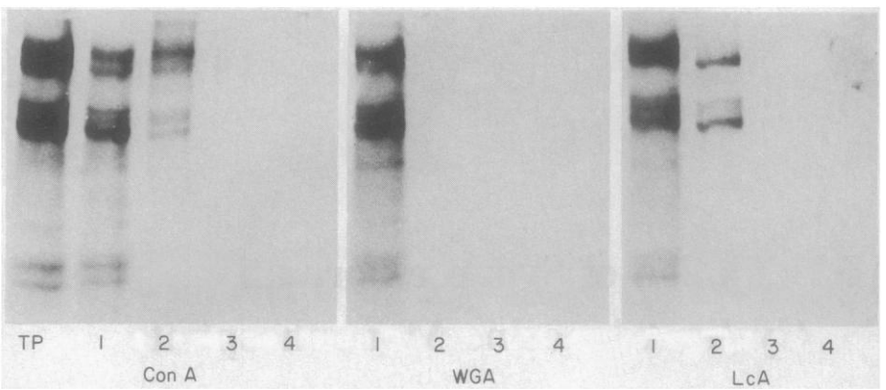
axons in a given fascicle from axons in neighboring groups.

The general organization of axon fascicles is symmetrical and stereotyped. A conclusion we have confirmed using the tracer enzyme horseradish peroxidase to locate the axons of physiologically identified individual neurons in the connective. For example, the axons of the mechanosensory cells were shown to run together (*9*). This observation that axons of physiologically similar cells run together is consistent with the observation of Stretton and Kravitz (*11*) who showed that functionally similar groups of axons run in stereotyped fascicles in the neuropil of the lobster.

In simpler invertebrates with fewer axons a stereotyped organization of the nerve cord is also found. The anatomy of the nematode nerve cord is known in detail (*12, 13*) and identified axons are found in symmetrical and stereotyped locations. In nematodes most axons do not branch and have fixed lengths; it follows from these facts that the position of the axon plays an important role in determining the possible synapses a neuron can make.

These observations and observations on other invertebrate species (*14–19*) show that axon location in the central nerve cord is determined, that physiologically distinct axons occupy specific locations and suggest that this organization plays an important role in establishing specific synaptic contacts between neurons.

In invertebrates, cellular studies on



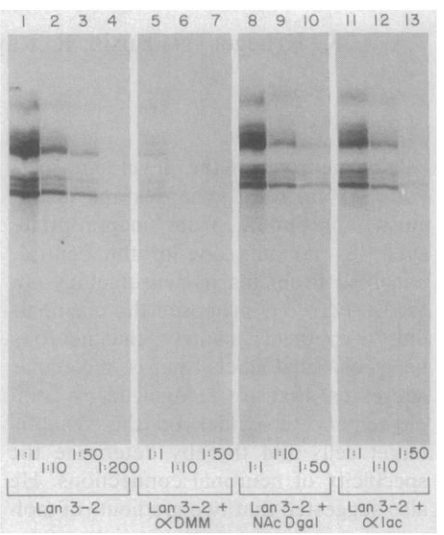


Fig. 7 (left). The Lan 3-2 antigens were glycosylated. Leech antigens were extracted from the dissected nerve cord by homogenizing in buffer A (10 percent Triton X-100, 150 mmole NaCl, 10 mmole tris-HCl, *p*H 8.0, 2 mmole EDTA, 1 mmole PMSF). Triton X-100 solubilized leech neural antigens were incubated with agarose beads covalently linked to the lectins Con A, WGA, LcA (LKB) for 1 hour on ice. The beads were then washed three times with buffer A (lane 1 shows unbound antigens) and eluted with 0.5 mmole sugar solutions (lanes 2 and 3, methylmannoside for Con A; *N*-acetyl-D-glucosamine for WGA; D-glucose for LcA). The sugar eluted beads were then boiled in a small sample of Laemmli buffer and samples of this elution were also run on a 10 percent polyacrylamide gel (lane 4). A sample of the total Triton-solubilized material is shown in the lane TP. These samples were transferred to nitrocellulose after electrophoresis and analyzed by immunoblotting with Lan 3-2 antibody. The major antigens recognized by Lan 3-2 bind to Con A and LcA agarose beads and are specifically eluted with sugar. Fig. 8 (right). Lan 3-2 binding is blocked by methylmannoside. Increasing dilutions of Lan 3-2 as marked gave a progressively weaker signal on Western blots (lanes 1, 2, 3, and 4). This binding of Lan 3-2 is markedly reduced in the presence of 500 m*M* methylmannoside (lanes 5, 6, and 7) but is unaltered by 500 m*M* *N*-acetyl-D-galactosamine or α-lactose (lanes 8 to 13). The conditions for the extraction and immunoblotting of leech antigens were as in Fig. 6.

the axonal development of identified cells in many different species shows that specific neurons recognize specific central axon tracts and regions of the neuropil. These studies suggest that a process of selective fasciculation mediated by cell contact is responsible for the specific arrangement of neuronal processes. In recent work, it has been shown that the filopodia of individual neurons can correctly distinguish certain axon fascicles (*20*).

Our data provide direct evidence for the presence of chemical differences on the surfaces of axon fascicles in the adult and in the embryo. These antigenic differences are present on the filopodia and are carried on a family of protease-sensitive, high molecular weight glycoconjugates. We do not yet know whether the particular antigens we have described are themselves directly involved in the mechanisms that guide selective axon fasciculation, but the fact that these molecular differences are present from early embryonic stages is consistent with the possibility that molecular differences between axon fascicles are responsible for the elaborate and precise geometry of axon outgrowth.

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Modulation of Synapse Formation by Cyclic Adenosine Monophosphate

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How neurons in the developing nervous system form synapses and distinguish appropriate from inappropriate synapses remains one of the central, unsolved problems in neurobiology. In 1963, Sperry (*1*) proposed the chemoaffinity hypothesis; namely, that neurons bear positional labels (that is, molecular addresses) that are recognized by complementary molecules on the synaptic target cells and thereby determine the specificity of neuronal connections. He also suggested that two gradients of molecules on retina neurons at right angles to one another, which interact with complementary molecules on the target neurons in the tectum, might be a mechanism for matching synaptic connections and reproducing a point-to-point map of the retina in the tectum. If synapse recognition molecules exist, monoclonal antibody technology should be a powerful tool for their detection. Many investigators are now using this approach.

Other mechanisms such as regulation of gene expression by environmental factors such as hormones, neuromodulators, transynaptic communication, or molecules secreted by neighboring or other cells surely play important roles in the assembly of synaptic circuits. For example, Le Douarin (*2*) and Patterson (*3*) and their colleagues have shown that during development neurons from the neural crest can express either the gene for tyrosine hydroxylase, which catalyzes the first step in the pathway for norepinephrine synthesis, or the gene for choline acetyltransferase, which catalyzes the synthesis of acetylcholine, depending on the presence of an extracellular macromolecule, purified by Weber (*4*), which is secreted by other cells, or the extent of depolarization of the neuron. In addition, Mudge (*5*) has shown that the expression of somatostatin, a peptide transmitter or neuromodulator, by dorsal root ganglia sensory neurons is dependent on molecules secreted by nonneural cells. Raff *et al.* (*6*) also have shown that fetal calf serum markedly influences the differentiation pathway expressed by glial cells in the central nervous system.

Edelman and his colleagues (*7*) discovered a neuronal glycoprotein rich in sialic acid residues, termed N-CAM (neural cell adhesion molecule), that mediates intercellular adhesion in the absence of Ca^{2+} and probably plays an important role in the development of the nervous system by conserving the topographic relationships between individual neurons or axons (or both) in a set of neurons, even though axons may migrate long distances before synapsing. Molecules that mediate Ca^{2+}-dependent intercellular adhesion (*8*) and factors that promote retina cell adhesion, such as cognin (*9*), and ligand and agglutinin (*10*), also have been described, but little is known about their function in the nervous system. Other mechanisms such as contact guidance, chemotaxis, cell survival factors, guidance of neurites by glia (*11*), and

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