Tract Formation and Axon Fasciculation of Molecularly Distinct Peripheral Neuron Subpopulations during Leech Embryogenesis

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Summary

In leech, the central projections of peripheral sensory neurons segregate into specific axonal tracts, which are distinguished by differential expression of surface antigens recognized by the monoclonal antibodies Lan3-2 and Lan4-2. Lan3-2 recognizes an epitope expressed on axons that segregate into three distinct axon fascicles. In contrast, the Lan4-2-positive axons selectively project into only one of the Lan3-2-positive axon tracts. These observations provide evidence for a hierarchy of guidance cues mediating specific pathway formation in this system. Since the Lan3-2 antibody has been shown to perturb this process and since, as shown here, the Lan3-2 and Lan4-2 antigens are closely molecularly interrelated, these antibodies may help define molecules and epitopes mediating neuronal recognition and axonal guidance.

Introduction

For precise neuronal connections to be established during early development axons must pioneer novel pathways through previously uncharted embryonic landscapes, both within the CNS and to and from the periphery. These pathways in turn may serve as guides for later differentiating neurons (Harrelson and Goodman, 1988; Raper et al., 1983; Dodd et al., 1988; Ghosh et al., 1990; Stanier and Gilbert, 1990), the axons of which often make highly specific pathway choices and show selective fasciculation. One proposed functional role of selective fasciculation is to guide the growth cones of related neurons into the vicinity of their proper common synaptic targets where specific contacts can then be made (McKay et al., 1983a). Most hypotheses about the molecular mechanism for selective fasciculation predict specific adhesion or recognition events between axons and/or growth cones mediated by surface macromolecules (for reviews see Harrelson and Goodman, 1988; Dodd and Jessell, 1988).

Cell surface proteins identified to date that may be involved in axon fasciculation fall into two classes: molecules that appear to play a general role in neural cell adhesion, for example, neural cell adhesion molecules, N-cadherins, and integrins (Jessell, 1988), and molecules that tend to be restricted to axonal surfaces, such as L1 (Moos et al., 1988), neurofascin (Rathjen et al., 1987), F11 (Brümmendorf et al., 1989), and contactin (Ranscht, 1988) and even more specific glycoproteins like TAG-1 (Dodd et al., 1988; Furley et al., 1990) and fasciclin I-III (Harrelson and Goodman, 1988; Zinn et al., 1988; Patel et al., 1987), which have restricted localization to subsets of axons. In recent years, molecular analysis of many of these glycoproteins has revealed striking common structural features and has in turn defined new gene families. For example, L1, TAG-1, neural cell adhesion molecule, contactin, and fasciclin II have all been shown to belong to the immunoglobulin supergene family and to contain multiple immunoglobulin-like domains (Harrelson and Goodman, 1988; Dodd and Jessell, 1988; Furley et al., 1990). Thus, common structural motifs in many functionally related molecules have been conserved in both invertebrates and vertebrates. However, it has been difficult to elucidate how these molecules mediate the recognition and formation of specific axon pathways or how many molecules and gene families involved in these events may exist. This is partly a function of the limited number of molecules that have been analyzed and the difficulty of designing good functional assays. Also, most of the molecules studied thus far have been amenable to analysis because they are relatively widely distributed and therefore are likely to be less specific and to play a more general role in axon fasciculation. One challenge is, therefore, to extend the structural molecular analysis of axon-specific surface molecules to those with as restricted a distribution as possible.

In the present paper, we examine the molecular properties and developmental expression of two antigens recognized by the monoclonal antibodies Lan3-2 and Lan4-2 (Zipser and McKay, 1981; McKay et al., 1983a). These antigens have a very specific expression, which is confined only to a small subpopulation of axon fascicles in the nerve roots and interganglionic connectives of the leech nervous system. These fascicles are formed exclusively by peripheral neurons projecting their axons into the CNS (McKay et al., 1983a; Peinado et al., 1990). The antigens are surface glycoproteins (McKay et al., 1983a, 1983b) and were originally identified and analyzed in the leech Haemopis marmorata. On Western blots of CNS extracts from this leech, Lan3-2 recognizes three protein bands with molecular masses of 130, 105, and 90 kd, respectively, whereas Lan4-2 recognizes only a single 130 kd band (McKay et al., 1983a). Several lines of evidence including regeneration and antibody perturbation experiments have provided strong support for the hypothesis that the Lan3-2 antigen is a surface glycoprotein directly involved in fascicle formation (McKay et al., 1983a; Johansen et al., 1985; Peinado et al., 1987a, 1990; Zipser et al., 1989).

Our present results with immunocytochemistry and
light and electron microscopy in Hirudo medicinalis demonstrate that the Lan3-2 antibody is likely to recognize all the developing peripheral neurons and that in embryonic day 10 (E10) embryos the central projections of their axons segregate into three separate axon fascicles. In contrast, the Lan4-2 antibody at this stage recognizes only a few peripheral neurons that also send axons to the CNS; however, their projections are confined to extend along only one of the Lan3-2-positive axon fascicles. Thus, more than one set of molecularly distinct guidance cues are likely to be involved in the normal fasciculation of these peripheral neurons. Furthermore, Western blot analysis of immunoprecipitations of CNS extracts with Lan3-2 and Lan4-2 antibodies demonstrates that the two antibodies recognize different epitopes on molecularly interrelated 130 kd antigens. Consequently, our results taken in conjunction with previous experiments (McKay et al., 1983a; Peinado et al., 1987a; Zipser et al., 1989) strongly suggest that the Lan3-2 and Lan4-2 antigens are molecules mediating selective axon fasciculation and neuronal recognition and demonstrate that multiple guidance cues are likely to be involved in this process.

Results

The Lan3-2 and Lan4-2 monoclonal antibodies label axons that form specific axonal tracts in all leech species so far examined (seven species representing two completely different orders of leeches; data not shown). This conservation of both epitopes over a phylogenetically broad spectrum suggests they are functionally important. In the present paper, we examine the biochemical properties and developmental expression of the Lan3-2 and Lan4-2 antigens in two genera of leeches, Haemopis and Hirudo. We chose Haemopis because this was the leech the antibodies were originally raised against and Hirudo because in contrast to Haemopis it can be bred year round in the laboratory. Immunocytochemically stained whole mounts (Figure 1A) demonstrate that similar axonal tracts in the CNS are labeled by the antibodies in both species. Notice that Lan3-2 in Haemopis also recognizes the cell bodies of the two pairs of medial and lateral nociceptive neurons, whereas Lan4-2 only recognizes the lateral pair (McKay et al., 1983a; Johansen et al., 1984). In all other leech species examined, including Hirudo, no central somata are labeled by either antibody.

The antibody-positive axons are derived from peripheral neurons (McKay et al., 1983a; Peinado et al., 1987a, 1990) located in the sensory sensilla and body wall as diagrammed in Figure 1B. The sensilla are clusters of mixed sensory neurons composed of chemoreceptors, photoreceptors, and mechanoreceptors (Phillips and Friessen, 1982) found on the central annulus of each segment (Muller et al., 1981). The sensilla are termed S1–S7, with the most ventral sensillum closest to the CNS in each hemisegment designated S1. S1–S5 sensillar neurons send their axons toward the CNS through the anterior nerve root whereas S6 and S7 neurons extend their axons through the posterior nerve root (Figure 1B) with one notable exception (see below). In addition, numerous neurons scattered throughout each body wall segment and the gut extend axons through these nerves into the central ganglia. Light and electron microscopic cross sections of antibody-labeled connectives have demonstrated that the Lan3-2 and Lan4-2 antigens both are surface antigens expressed on tightly bundled axons found in similar stereotyped positions (diagrammed in Figure 1C) in the lateral connectives (Hockfield and McKay, 1983; McKay et al., 1983a). After axogenesis and fascicle formation the bundles are wrapped by process invaginations from a single glial cell in each connective (Morrissey and McGlade-McCullough, 1988). In adult leeches this obscures the original spatial relationship between the embryonic axons and fascicles.

Development of Peripheral Sensilla and Axon Fascicles

In the present study we have used the Lan3-2 and Lan4-2 antibodies as markers for peripheral neurogenesis and early fascicle formation in Hirudo. In leech the formation of both the central and peripheral nervous systems proceeds in a rostro-caudal sequence (Stent et al., 1982). Generally, each posterior segment is approximately 2 1/2 hr later in development than its immediate anterior segment. Consequently, since there are 32 segments, an E10 embryo exhibits segments in different stages of development spanning a period of about 2–3 days, which corresponds to 10% of total development.

In addition to the rostro-caudal progression our results demonstrate that the sensilla also arise within a given segment in a distinct pattern that does not, however, follow a simple sequence. Surprisingly, one of the middle sensilla, S3, is the first sensillum to be detected by Lan3-2. The primordium initially (Figures 2A and 2B) consists of a few cells that send tightly associated axons with numerous growth cones toward the CNS. The first sensillar primordia are detectable in the anterior segments of E8 embryos (Figure 2A). When the growth cones of these axons reach the central neuropil, they execute a sharp turn and establish three separate axon tracts (Figure 3; Figure 4) by extending filopodia anteriorly and posteriorly without crossing the midline of the ganglion (Figure 4). This change of direction is not random but rather takes place at a stereotypic location in each ganglion. Neurons in S7 and S6 appear at about the time when the S3 growth cones reach the CNS. As shown in Figure 2C, the growth cones of the most dorsal sensilla, S6 and S7, pioneer a completely different pathway toward the CNS than the ones of S3. Subsequently, sensillar neurons S1, S2, S4, and S5 appear. The S1, S2, S4, and S5 sensillar afferent axons all follow and fasciculate with the S3 axons that preceded them. Although the S6 and S7 afferents navigate a different route to the CNS they also extend into the tracts pre-
Figure 1. Lan3-2 and Lan4-2 Labeling of Peripheral Neuron Axonal Tracts

(A) Lan3-2 and Lan4-2 label similar subsets of axonal tracts in adult ganglia of Hirudo and Haemopis. Anterior is to the left. Bar, 150 μm.

(B) Diagram of peripheral neurons that contribute to the antibody-positive axonal tracts in (A). One hemisegment is shown. There are seven groups of sensory neurons constituting the sensilla, which are labeled S1–S7. The axons from S1–S5 enter the ganglion through the anterior nerve; the axons from S6 and S7 enter through the posterior nerve. Not included in this diagram is the single Lan4-2-positive neuron located in S6 that extends its axon through the anterior nerve. In addition to the sensillar neurons, various unidentified neurons scattered in the body wall (open circles) also contribute antibody-positive axons to the tracts. These neurons, in contrast to the sensillar neurons, appear late in embryogenesis. (C) Diagram of a cross section of a connective (see [B]) illustrating the stereotyped position of the antibody-positive axonal tracts. The interganglionic connective is composed of a pair of lateral nerves (LC) and an unpaired medial nerve, Faivre’s nerve (F). The appearance of multiple axon tracts in (A) and (C) is a consequence of the extensive glial invaginations, which obscure in adult leeches the original spatial relationship of the embryonic axons and fascicles.
Figure 2. Early Development of Lan3-2 and Lan4-2 Sensillar Neurons

(A) The sensillar neurons that appear earliest labeled by Lan3-2 in the anterior part of an E8 embryo. The primordium consists of three to five neurons that extend bundled axons toward the CNS. Bar, 25 μm.

(B) S3 sensillar neurons labeled by Lan3-2 in a posterior segment of an E10 embryo. At this stage only S3 neurons are antibody positive, and their axons have just reached the ganglion. The growth cones are in the process of executing a sharp turn of direction in order to establish rostrally and caudally oriented axonal tracts. The axons of all the S3 neurons are tightly fasciculated. Bar, 25 μm.

(C) Progressive stages of the formation of a pathway to the CNS through the posterior nerve by sensillar neurons from S6 and S7. Arrows point to the tip of the growth cones at each stage. Bar, 35 μm.

(D-F) Development of sensillar neurons labeled by Lan4-2. Bar, 25 μm. (D) shows the earliest primordium of a single Lan4-2-positive neuron before an axon is extended in the posterior segments of an E10 embryo. This neuron is located in a position where the S6 sensillum eventually will differentiate. At later stages an axon is extended (E), and subsequently several neurons with similar morphology are Lan4-2 positive within each sensillum (F).

(G) shows, at a similar stage as (E), the entire projection of the first Lan4-2-positive neuron that extends a single axon into the ganglion (g). Bar, 60 μm.
Figure 3. Axonal Tracts Labeled by Lan3-2 and Lan4-2 in E10 Sibling Embryos

(A and B) Whole-mount preparations of two ganglia (micrbody ganglia 3 and 4) linked by the interganglionic connective labeled with Lan3-2 and Lan4-2. Lan3-2 labels three distinct tracts, although in whole mounts generally only two tracts are clearly visible due to their relative spatial position. Lan4-2 labels only one fascicle. Bar (A and B), 60 μm.

(C and D) Cross sections of connectives between ganglia 3 and 4 from sibling E10 embryos labeled by Lan3-2 and Lan4-2, respectively. In (C) the three tracts labeled by Lan3-2 are clearly visible, whereas Lan4-2 labels only one tract (D), which is copositional with the most lateral Lan3-2-positive fascicle. Bar (C and D), 8 μm.

(E and F) Electron micrographs of cross sections of one of the lateral connectives between ganglia 3 and 4 from sibling E10 embryos labeled by Lan3-2 and Lan4-2. The antibody-stained tracts are marked by the adjacent asterisks. The developing single glial cell (gl) that wraps the axons is just beginning to extend its processes at this stage. Numerous growth cone filopodia are present between the axon profiles. Bar (E and F), 2.5 μm.

Previously established in the ganglia and connectives by the S3 axons.

Selective Tract Formation by a Subset of Peripheral Neurons Recognized by Lan4-2

The Lan3-2 antibody is likely to recognize all early developing peripheral sensory neurons (Peinado et al., 1987a) although this notion cannot be directly demonstrated. It is clear, however, that the Lan4-2 antibody labels only a distinct subset of the Lan3-2-positive neurons (Figure 3; Figure 4; Figure 5B). Early in development Lan4-2 labels a single peripheral neuron in S6 that extends its axon toward the CNS (Figure 2G). This neuron is identifiable by a distinct morphol-
Figure 4. Comparison of Lan3-2- and Lan4-2-Positive Axon Fascicle Development in Sibling Embryos
Equivalent midbody ganglia (g4, g11, g15, g18) in each preparation were labeled with Lan3-2 and Lan4-2, respectively. The Lan4-2-positive neurons develop considerably later than the general population of Lan3-2-positive neurons. In g4 Lan3-2-positive axons have established three tracts, whereas the Lan4-2-positive subset of axons only extends into one tract. In g18 only Lan3-2-positive axons have entered the ganglion. The arrowhead points to the growth cones of S6 and S7 Lan3-2-positive sensilla axons that have just reached the neuropil of the ganglion through the posterior nerve in g15. The primordial axon tracts established by the S3 axons are indicated by arrows in this micrograph. Bar, 30 μm.
Figure 5. Double Labels with Lan3-2 and Lan4-2 of Sensillar Neurons and Their Axons in the PNS and CNS of an E10 Embryo (A) shows the single S6 sensillar neuron labeled by Lan4-2 at this stage of development. The white arrow points to this cell's axon (brown reaction product), which instead of following the posterior nerve (pn) enters the CNS through the anterior nerve (an). This figure also demonstrates that numerous sensillar neurons labeled by Lan3-2 (blue reaction product) have differentiated at this time and extended their processes toward the CNS through both nerves. The Lan4-2-positive neuron also expresses the Lan3-2 epitope (see text); however, the first antibody labeling appears either to inhibit the second labeling or prevent the blue reaction product from forming, wherefore very little blue reaction product is visible on this axon under these conditions. Bar, 25 μm. (B) shows that the Lan4-2-positive axons project into only one of the Lan3-2-positive fascicles. The most lateral fascicle (black arrows) consists of Lan4-2-positive axons (brown reaction product); the fascicle clearly is also associated with blue reaction product from the additional labeling of Lan3-2-positive axons (white arrows). In contrast, the innermost visible fascicle is labeled only by Lan3-2 (blue reaction product, arrowheads). Bar, 25 μm.

ogy having many short processes extending from the cell body (Figures 2D and 2E). This neuron is further distinguished by the observation that although its cell body is located in S6, its axon will enter the CNS through the anterior nerve as demonstrated in a double-labeled whole-mount preparation (Figure 5). The embryo was first labeled with Lan4-2 using horse-radish peroxidase (HRP)-conjugated second antibody (brown reaction product) and thereafter with Lan3-2 using a phosphatase-conjugated second antibody (blue reaction product). This procedure was necessary since both antibodies are of the same immunoglobulin G1 subtype. The binding of Lan4-2 antibody apparently prevents or obscures the labeling with Lan3-2 so that the Lan4-2-positive axons are only labeled brown, not brown and blue. However, it is clear that at this stage, Lan4-2 recognizes neurons that develop with a considerable delay compared with the population of sensillar neurons labeled by Lan3-2. The earliest appearance of Lan4-2-positive neurons is in the anterior segments during E9–E10, a time when the S3 neuronal axons have already reached the CNS in these segments. However, the first Lan4-2-positive neuron in S6 precedes the differentiation of the other Lan3-2-positive neurons in this sensillum. In all cases, these Lan4-2-positive neurons are also labeled by Lan3-2. Figures 2D–2F show the sequence of differentiation of the Lan4-2-positive neurons. At later stages, several neurons within all the sensilla are stained by Lan4-2 (Figure 2F). The axons of these later appearing neurons in S6 and S7 travel through the posterior nerve rather than fasciculating with the first Lan4-2-positive neuron in S6 that extended through the anterior nerve.

When the Lan4-2-positive axons reach the CNS, three axonal tracts have already been established by the other sensillar neurons (Figure 3; Figure 4). However, the Lan4-2-positive axons select only one of these fascicles (Figure 3; Figure 5). To illustrate the temporal relationship during development between the total Lan3-2-positive neuronal population and the Lan4-2 neurons (Figure 4) we dissected synchronously developing siblings from E10 cocoons and stained half with Lan3-2 and the other half with Lan4-2. This al-
allowed us to compare the degree of fascicle formation between ganglia of equivalent development even though they were from different preparations. At early stages, in this case exemplified by midbody ganglion 18 (Figure 4, g18), the S3 growth cones stained by Lan3-2 have reached the CNS and begun extending elaborate filopodia within the ipsilateral neuropil. No Lan4-2-positive axons have yet reached the CNS at this stage. However, in a slightly more advanced segment (Figure 4, g13) the first Lan4-2-positive axon has just reached the CNS, whereas many of the Lan3-2-positive axons have already made their turn and extended processes into the anterior and posterior interganglionic connectives. Some of the axons from S6 and S7 (Figure 4, arrow) are poised to enter the neuropil, having extended through the primordial posterior nerve. In ganglion 11 (Figure 4, g11), an even more advanced stage, the Lan3-2-positive neurons have established distinct axonal tracts in the connectives extending continuously between ganglia. In contrast, at this time the Lan4-2-positive axons are just about to change their direction of growth within the CNS and to extend neurites into the connectives. In one of the anterior and most differentiated ganglia (Figure 4, g4) numerous axons are fasciculated in three distinct tracts (Figure 3), and the single Lan4-2-positive tract also extends between ganglia. At this more advanced stage Lan4-2-positive CNS tracts are made up of several axons, including those from the dorsal sensilla S6 and S7.

Figures 3C and 3D show the spatial relationship between the Lan3-2- and Lan4-2-positive axon tracts in cross sections of E10 sibling preparations sectioned between ganglia 4 and 5. Lan3-2-positive axons travel in three separate tracts whereas Lan4-2-positive axons are localized into only one tract (Figure 3). The position of the Lan4-2 tract corresponds to the most lateral tract labeled by Lan3-2. This was directly demonstrated with a double-labeling experiment using Lan3-2 and Lan4-2 (Figure 3B). In this figure the most lateral of the axon tracts labeled by Lan4-2 and Lan3-2 are partly brown and partly blue, whereas the medial tract labeled by Lan3-2 is only blue. Thus, as predicted from our results with sibling embryos, the Lan4-2-positive axons travel in the same fascicle as the most laterally positioned Lan3-2-positive axons. Furthermore, in cross sections processed for electron microscopy it is clear that these fascicles have formed before the axons are wrapped in glial involutions (Figures 3E and 3F). Another feature in these micrographs is that among the labeled axons several smaller profiles are seen that probably correspond to growth cone filopodia. Although the HRP product is somewhat overdeveloped in these preparations to ensure good visibility only about ten individual Lan4-2-positive axons are discernable in contrast to the several dozen labeled by Lan3-2.

**Sensory Afferents in Later Stages of Embryonic Development**

At later stages (E12–E13) a fourth Lan3-2-positive fascicle develops in the CNS (K. M. Johansen, D. M. Kopp, J. Jellies, and J. Johansen, unpublished data; Zipser et al., 1989). The fascicles are characterized during their formation by the presence of elaborate growth cones (Figure 3; Figure 4) both in the neuropil where the turn is made and along the interganglionic tracts. However, the axons of later differentiating peripheral neurons do not exhibit such extensive growth cones as those extended when the tracts are pioneered.

An interesting feature of PNS development in leech is that the number of sensory afferents continues to

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**Figure 6. Late-Appearing Extra Sensillar Neurons Labeled by Lan3-2 in the Body Wall of an E25 Embryo**

(A) Low power micrograph of extra sensillar Lan3-2-positive neurons in the body wall. A ganglion (g1) is in the lower right-hand corner. Bar, 150 μm. (B) Example of relatively large neurons in the body wall labeled by Lan3-2. Bar, 40 μm. (C) A neuronal type different from the one in (B), which has an irregularly shaped soma (large arrowhead). Small arrowheads indicate a parallel row of a third, smaller kind of neuron labeled by Lan3-2 that is slightly out of focus in this picture. Same magnification as in (B).
increase throughout the lifespan of the leech (Peinado et al., 1990). By antibody labeling we have identified a population of peripheral neurons in the skin that differentiate relatively late in embryonic development, around E25, a few days before hatching. Figure 6 shows embryonic skin preparations from this stage of development stained with Lan3-2. All labeled neurons are extending axons toward the CNS. At least three morphological types of neurons could be differentiated by the antibody labeling; a neuron with relatively large, round soma and a large "dendritic spine" (Figure 6B), a smaller neuron with a round soma often appearing in parallel rows (Figure 6C, small arrowheads), and a larger neuron with an irregularly shaped soma (Figure 6C). All these neurons were also labeled in a similar fashion by Lan4-2. These neurons are more numerous than sensillum neurons and eventually contribute the majority of axons in the sensory afferent fascicles. Embryonically late-differentiating afferent neurons stained by Lan3-2 have also been reported in Helobdella (Johansen et al., 1985), and similar neurons labeled by another antibody, Lan3-6, which also labels peripheral sensory neurons, have been described in Haemopis (Stewart et al., 1985).

**Molecular Interrelatedness of the Lan3-2 and Lan4-2 Antigens**

Given that Lan4-2-positive peripheral neurons represent a subset of the Lan3-2-positive neurons and that both antibodies recognize epitopes expressed on 130 kD glycoproteins (McKay et al., 1983a), we investigated the biochemical relationship of the antigens and their epitopes in both Haemopis and Hirudo. Figure 7A shows an immunoblot comparison of CNS extracts from Haemopis and Hirudo labeled with Lan3-2 and Lan4-2, respectively. This analysis reveals several notable features. In Hirudo and Haemopis both antibodies recognize a relatively broad 130 kD band as previously reported (McKay et al., 1983a; Flanagan et al., 1986; Bajt et al., 1990). However, Lan3-2 also recognizes two lower bands in Haemopis. These have different apparent molecular weights than the three lower bands recognized in Hirudo (Figure 7A), and none of the lower bands is labeled by Lan4-2 in either leech. The number of proteins having different molecular weights labeled by Lan3-2 suggests that considerable heterogeneity may exist between the antigens on the molecular level of the different species even though the immunocytochemical staining (Figure 1A) is very similar. This observation is further supported by Figure 8, which shows an immunoblot of CNS extracts from five leech species labeled with Lan3-2. Although many proteins of different molecular weights appear to be labeled in the different leech species, in all cases, a ~130 kD protein is present, suggesting the presence of a common core protein.

The finding that Lan3-2 and Lan4-2 in both Haemopis and Hirudo recognize a 130 kD protein raises the issue whether these antigens are molecularly interrelated. To address this question we immunoprecipitated the Lan3-2 antigens from Haemopis. The purified proteins from five leech nerve cords were then separated by
Lan3-2 and Lan4-2 epitopes are expressed on separate 130 kd proteins that coprecipitate due to strong mutual adhesion. In either case, our experiments demonstrate that the Lan3-2 and Lan4-2 antigens are molecularly interrelated and that the two epitopes are differentially expressed by a subset of peripheral neuron axons.

Another issue we addressed was a comparison of the molecular nature of the Lan3-2 and Lan4-2 epitopes. Since it is known that the Lan3-2 epitope is at least partly composed of carbohydrate (Mckay et al., 1983a), we tested whether this is also the case for the Lan4-2 epitope. We treated Western blots of SDS-PAGE-separated CNS extracts from Hirudo with N-glycanase, which cleaves off N-linked carbohydrates. As shown in Figure 7D, this treatment significantly reduced antibody labeling compared with untreated and mock-digested controls. It is therefore likely that the Lan4-2 epitope is also either partially or fully made up of carbohydrate.

**Discussion**

**Specific Axon Fascicle Formation during Early Embryogenesis**

In this paper, we have studied pathfinding and specific axon fascicle formation of peripheral neurons in the leech using two monoclonal antibodies, Lan3-2 and Lan4-2, as markers. The Lan3-2 antigen is likely to be expressed by the entire population of peripheral sensory neurons. The first Lan3-2 antibody-positive neurons differentiate in the primordium of the third sensillum in E8 embryos. These sensilla neurons project axons with growth cones directed toward the CNS, where they establish three rostrally and caudally oriented axon tracts in stereotyped locations. During subsequent differentiation of the seven groups of sensilla neurons, their afferents selectively grow and fasciculate along these primordial tracts. However, after these Lan3-2-positive tracts are established, a morphologically distinct population of sensilla neurons, which is specifically labeled by Lan4-2, differentiates. In contrast to the total population, which segregates into three tracts, these sensilla neurons selectively recognize and fasciculate with only one of the Lan3-2-positive tracts.

Although Lan3-2 Fab fragments have been shown to perturb the formation of the axon fascicles during development (Zipser et al., 1989), the fact that they segregate into three separate tracts in stereotyped locations appears to rule out that they are established by means of a simple homophilic adhesion mechanism involving the Lan3-2 epitope. Thus, these data suggest that several different guidance cues, which may interact in highly complex spatially and temporally regulated ways, are necessary for the correct segregation of these axons into distinct fascicles. A possible role for the Lan3-2 antigen in this scenario may be that it acts as a receptor transducing information from as yet unidentified ligands providing a hierarchy of molecu-
lar guidance cues (Jessell, 1988). That such a hierarchy may exist in this system is supported by the observation that other monoclonal antibodies in addition to Lan3-2 and Lan4-2 have been identified that can further distinguish between subsets of these sensory afferent fascicles (Peinado et al., 1987b, 1990), thus providing additional evidence that multiple subsets of axons carry markers that make them molecularly distinct. Recently, experiments with chromophore-assisted laser inactivation (Jay and Keshishian, 1990) and genetic analysis (Grenningloh et al., 1991) have directly demonstrated that fascicle-specific cell surface proteins are important for axonal adherence and pathway formation.

Molecular Relationship between the Lan3-2 and Lan4-2 Antigens

Immunoblot analysis of CNS proteins demonstrates that Lan3-2 and Lan4-2 both recognize a 130 kd cell surface antigen and that their epitopes are different and at least partly composed of carbohydrate. Immunoblot comparisons also show that in addition to the 130 kd glycoprotein, Lan3-2 recognizes a number of other molecular weight proteins in different leech species. The significance of these bands is presently unclear. In Hirudo differential phase separation experiments with Triton X-114 have shown that the lower bands have a different membrane association than the 130 kd protein (Bajt et al., 1990). Furthermore, immunoblots of embryonic CNS suggest that in E8-E10 embryos, where the Lan3-2-positive fascicles are being formed, only the 130 kd version of these antigens is expressed (McGlade-McCullough et al., 1990). Thus, these experiments suggest that the 130 kd protein may be the key molecular form of the Lan3-2 antigens involved in the formation of the axon fascicles, since it is the only form expressed when the fascicles are first pioneered.

Furthermore, our immunoblot analysis and immunoprecipitation experiments demonstrate that the Lan3-2 and Lan4-2 antigens are closely molecularly interrelated. On the basis of the current experiments there are two ways to account for their relationship. One possibility is that the Lan3-2 and Lan4-2 epitopes are expressed on separate 130 kd proteins that coprecipitate due to strong mutual adhesion, the Lan4-2 protein being expressed by only a distinct subset of peripheral neurons during early fascicle formation. The other possibility is that the Lan3-2 and Lan4-2 epitopes are expressed on the same molecule. In this case, all the peripheral neurons express a 130 kd protein carrying the Lan3-2 epitope whereas only a subset of these neurons express a 130 kd version of the protein that carries both the Lan3-2 and the Lan4-2 epitopes. While it is clear from our combined biochemical and immunocytochemical data that not all 130 kd Lan3-2-positive proteins would coexpress the Lan4-2 epitope, the Lan4-2 epitope may always be coexpressed with the Lan3-2 epitope. Thus, the 130 kd protein may be composed of a core protein that, either by differential mRNA splicing or by differential glycosylation, expresses different epitopes involved in selective fascicle formation.

At present our experiments do not allow us to distinguish between the two hypotheses outlined above. However, we plan to address this issue by applying our purification procedure to obtain enough antigen for partial amino acid sequence determination and cloning of the gene(s). Subsequent analysis of the gene products will definitively resolve the issue. In either case the experiments clearly demonstrate that the Lan3-2 and Lan4-2 epitopes are differentially expressed, both on subsets of axonal tracts and on molecules that are closely interrelated. While the Lan3-2 epitope has already been demonstrated to play a role in axon fasciculation (Zipsper et al., 1989), our data are suggestive of the possibility that the Lan4-2 epitope also in some way may be involved in axonal tract selection during early development.

Late-Differentiating Extrasensillar Peripheral Neurons Expressing the Antigens

In addition to the events of early fascicle formation we also present evidence that several morphologically different populations of extrasensillar peripheral neurons differentiate at late stages of development shortly before hatching. These neurons express both the Lan3-2 and Lan4-2 epitopes and fasciculate within the tracts of the sensillar afferents. The expression of the Lan3-2 and Lan4-2 antigens persists throughout the lifespan of the leech, not just in the critical early periods when fascicles are formed. This probably reflects that the number of peripheral neurons continues to increase with the increase in skin area as the animal grows (Peinado et al., 1990). This was further supported by axon counts on electron micrographs of nerve sections from adult leeches of different sizes that show that total axon number including sensory afferents increases with leech size (Peinado et al., 1990). A rationale for why these peripheral neurons travel together in distinct fascicles would be that the increasing numbers of peripheral neurons recruited with the growth of the leech are likely to be sensory neurons (Peinado et al., 1990) that may make synaptic contacts with common targets in the CNS. Throughout embryonic development and postembryonic maturation, guidance cues on the existing fascicles, which were pioneered when distances were short, would be critical for the correct navigation in the CNS of the axons of the peripheral neurons. Direct support for the notion that the peripheral neuron axon fascicles indeed carry some form of label that can direct fasciculation and neuronal recognition comes from regeneration experiments (Peinado et al., 1987a). If the nerve roots were crushed, almost all of the regenerating axons from neurons in the peripheral sensilla were found within the Lan3-2-positive tracts in the nerve roots and not elsewhere (Peinado et al., 1987a). This clearly indicates that the regenerating peripheral axons are preferentially guided to grow along specific tracts.
On the basis of these observations and the results of the present paper, we propose that the Lan3-2- and Lan4-2-positive antigens carry epitopes that may mediate selective axon tract formation of the peripheral axons, since, as summarized here, all their known properties are consistent with such a role: First, the Lan3-2 and Lan4-2 antigens define a small group of specific axon fascicles that occupy stereotyped positions in the connectives and nerve roots (McKay et al., 1983a; Hockfield and McKay, 1983). Second, the Lan3-2 and Lan4-2 antigens are present on the surface of axons (McKay et al., 1983a). Third, the antigens are expressed at early stages of neurite outgrowth and are present at growing cones. Fourth, the Lan3-2 and Lan4-2 antigens are closely molecularly interrelated and likely to represent either differential expression of different epitopes on a common core protein or expression of different epitopes on separate proteins with strong molecular interactions. Fifth, the epitopes of Lan3-2 and Lan4-2 are conserved in several leech species from two different orders. Sixth, Lan3-2-positive fascicles can guide the growth of regenerating peripheral sensory axons (Peinado et al., 1987a). Seventh, direct evidence suggests that fascicle formation during axogenesis can be perturbed by the Lan3-2 antibody (Zipser et al., 1989).

Our long range goals in analyzing and cloning the Lan3-2 and Lan4-2 antigens as well as other leech antigens (Briggs et al., 1991; Soc. Neurosci., abstract) specific for axons and axonal subsets are to gain basic insights into the functional significance of such molecules, their possible hierarchical organization, functional determinants, and developmental regulation of expression. As our results demonstrate, this relatively simple and experimentally accessible system promises to be useful for investigating interactions among molecules mediating axonal pathway choices.

Experimental Procedures

Experimental Preparations

Leech Species

For the present experiments we used several different leech species from two orders, namely the hirudinid leeches Hirudo medicinalis, Haemopis marmorata, and Macrobodella decora and the glossiphonid leeches Haementeria ghilianii and Placobdella parasiticae. The leeches were either captured in the wild or purchased from commercial sources.

Dissections

Dissections of nervous tissue and embryos were performed in leech saline solutions with the following composition: 110 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 10 mM glucose, 10 mM HEPES (pH 7.4). In some cases 8% ethanol was added, and the saline solution was cooled to 4°C to inhibit muscle contractions.

Embryos

Hirudo medicinalis embryos were obtained from a laboratory breeding colony as previously described (Jellies et al., 1987). Cocoons were maintained at 24°C, and embryos were staged according to the criteria described by Fernandez and Stent (1982). There are about 10-20 embryos in each cocoon, and these sibling embryos develop synchronously within a few percent of development.

Immunocytochemistry

Two monoclonal antibodies, Lan3-2 and Lan4-2 (Zipser and McKay, 1981; McKay et al., 1983a), were used in these studies. They were both determined to be of the immunoglobulin G1 subtype by DuChemin immunodiffusion assays.

Dissected Hirudo embryos were fixed overnight at 4°C in either 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), 2% glutaraldehyde/2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), or 95% ethanol with 5% (v/v) glacial acetic acid. Similar antibody labelling results were obtained with all three fixatives. For better antibody penetration the embryos were xylenextracted (Zipser and McKay, 1981) before antibody labelling. The embryos were incubated overnight at room temperature directly in hybridoma supernatant containing 0.4% Triton X-100, washed in phosphate-buffered saline with 0.4% Triton X-100, and incubated with HRP-conjugated goat anti-mouse antibody (BioRad, 1:100 dilution). After washing in phosphate-buffered saline the HRP-conjugated antibody complex was visualized by reaction in 3,3′ diaminobenzidine (0.03%) and H₂O₂ (0.01%) for 10 min. The final preparations were dehydrated in alcohol, cleared in xylene, and embedded as whole mounts in Depex mountant.

Adult ganglia were fixed in 4% paraformaldehyde, and the connective capsules were opened with fine forceps before they were processed for antibody labeling in a similar way to the embryos. For double labelings the embryos were first incubated with Lan4-2 antibody and processed with HRP-conjugated secondary antibody as described above. This results in a brown reaction product. After this first step the preparations were incubated in the Lan3-2 antibody, the labeling of which was visualized using an alkaline phosphatase-conjugated secondary antibody (BioRad, 1:150 dilution). For alkaline phosphatase labelings the embryos were transferred to 1 ml of phosphate-buffered saline (pH 9.5) with 100 μM levamisole (Sigma) and reacted by adding 3.5 μl of 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide and 4.5 μl of 75 mg/ml Nitro Blue Tetrazolium in 70% dimethylformamide. This gives rise to a blue reaction product easily distinguishable from the brown HRP reaction product. After the double labeling the embryos were embedded as whole mounts.

Immunoelectron Microscopy

For electron microscopy embryos were fixed in 2% glutaraldehyde/2% paraformaldehyde in 0.1 M phosphate buffer for 1 1/2 hr in 1% sodium borohydride (Elred et al., 1983) before antibody labeling. In these experiments xylene extraction and Triton X-100 in the buffers were omitted in order to preserve ultrastructural integrity as much as possible. The preparations were labeled by Lan3-2 and Lan4-2, respectively, using HRP-conjugated secondary antibody for visualization. After antibody labeling the embryos were postfixed in 2% OsO₄, for 1 hr and embedded in epoxy resin (Durcupan). Blocks were sectioned on a Reichert Ultracut E microtome with either glass or diamond knives. Thick sections of connective tissue were cut at 2 μm and photographed through a Leitz light microscope. Thin sections of 90–110 nm thickness were collected on Formvar-coated slot grids, stained with 1% Knoxo for 15 min and lead citrate for 10 min, and examined with a Jeol 100CX electron microscope.

Biochemical Analysis

Lectin Affinity Chromatography

Enrichment for the Lan3-2 and Lan4-2 antigens was achieved by selecting for the glycoprotein fraction using a lentil lectin-Sephrose column. Dissected nerve cords were homogenized in lentil lectin binding buffer (20 mM Tris–HCl, 200 mM NaCl, 2 mM CaCl₂, 0.2% Nonidet P-40, 0.2% Triton X-100 (pH 7.4)), containing protease inhibitors. This homogenate was then batch incubated overnight at 4°C with lentil lectin–Sephrose beads. The beads were poured into a column; the column was washed with lentil lectin binding buffer, and the bound fraction was competed off with two 15 min incubations with 25 mM Tris–HCl, 10% methyl o-mannopyranoside, 0.15% SDS (pH 7.4).

Immunoadfinity Purification

The Lan3-2 and Lan4-2 antigens were partially purified by incubating the antigens with the antibodies followed by immunoprecipi-
tation using immunobeads coupled to rabbit anti-mouse antibody Bio-Rad. This procedure circumvents the problem of poor binding of protein A to the mouse immunoglobulin G subclass, yet enables the antibody-antigen complex to be spun down using an analogous technique. First, dissected nerve cords were homogenized in extraction buffer as for lectin affinity chromatography. This homogenate was precleared in preparation for immunoprecipitation by incubation in rabbit anti-mouse-coupled immunobeads in order to remove any antigens that nonspecifically adhere to the immunobeads. The immunobeads were spun down, and the supernatant was immunoprecipitated by incubation with Lan3-2 or Lan4-2 hybridoma supernatant. The antibody-antigen complex was then bound to the rabbit anti-mouse-conjugated immunobeads. After centrifugation and stringent washes, the bound antibody-antigen complex was separated from the beads by boiling in SDS sample buffer, yielding pure proteins and the antibody heavy and light chains. In addition, some contamination from serum albumin from the hybridoma supernatant is unavoidable in this procedure; however, due to the lower molecular weight of these molecules they do not interfere with the purification of the higher molecular weight Lan3-2 and Lan4-2 antigens.

SDS-PAGE and Western Blotting

SDS-PAGE was performed according to standard procedures (Laemmli, 1970) except that acrylamide was used at 10% and bisacrylamide at 0.13% (this recipe gave better resolution of the Lan3-2 and Lan4-2 antigens). Electrophorese transfer was performed, as in Towbin et al. (1979). For these experiments we used the Bio-Rad minigel system, electroblotted to nitrocellulose, and used HRP-conjugated secondary antibody (1:3000) for visualization of primary antibody in immunoblot analysis. The signal was developed with 3,3'-diaminobenzidine (0.1 mg/ml) and H2O2 (0.03%) and enhanced with 0.008% NiCl2. Gels were fixed and silver stained using the Bio-Rad silver stain kit as per the manufacturer's instructions.

N-Carboxyamidination

SDS-PAGE and electroblotting was performed as described above, with lanes run in triplicate. After electroblotting, proteins were visualized by staining the nitrocellulose in Ponceau S (0.3% in 2% (v/v) acetic acid) and destained in distilled water. Lanes were then cut into individual strips. One such strip in the triplicate was processed as above for Western blot antibody detection of the Lan4-2 antigen. The Lan4-2-positive regions of the other nitrocellulose strips were then excised from the unprocessed lanes, placed into a 24-well microtiter dish, and either digested with N-glycanase (Endo) or mock digested (identical treatment except no enzyme was added) according to the manufacturer's instructions. Essentially, reactions were performed in 0.5 mL reaction volumes in 0.2 M sodium phosphate, 1.25% Nonidet P-40, 0.1% SDS, 0.04 M β-mercaptoethanol (pH 8.6) with 1 U of N-glycanase added in the experimental samples. Samples were incubated overnight at 37°C with shaking, washed, and processed for antibody detection as done for the untreated nitrocellulose strips.

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