

Developmental expression of the lipocalin Lazarillo and its role in axonal pathfinding in the grasshopper embryo

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SUMMARY

This article describes the expression pattern and functional analysis of Lazarillo, a novel cell surface glycoprotein expressed in the embryonic grasshopper nervous system, and a member of the lipocalin family. Lazarillo is expressed by a subset of neuroblasts, ganglion mother cells and neurons of the central nervous system, by all sensory neurons of the peripheral nervous system, and by a subset of neurons of the enteric nervous system. It is also present in a few non neuronal cells associated mainly with the

excretory system. A monoclonal antibody raised against Lazarillo perturbs the extent and direction of growth of identified commissural pioneer neurons. We propose that Lazarillo is the receptor for a midline morphogen involved in the outgrowth and guidance of these neurons.

Key words: axon guidance, lipocalin, grasshopper, lipid binding proteins

INTRODUCTION

Among the more intriguing issues that developmental neurobiologists have faced is the generation of the enormous cellular diversity in the nervous system and the mechanisms that define how these diverse cells establish the intricate network of neuronal connections. A general strategy for understanding neurogenesis and axon pathfinding has been to examine, both at the cellular and molecular level, the less complex nervous systems of invertebrates. The central nervous system (CNS) in insects develops from a monolayer of ventral ectodermal cells called the neurogenic region where a set of enlarged cells, the neuroblasts (NBs), differentiate in a stereotyped number and position. After their differentiation, NBs undergo several oriented asymmetric divisions giving rise to smaller ganglion mother cells (GMCs) that will divide symmetrically to generate two postmitotic neurons. The stereotypy and invariance displayed by the progeny (Doe et al., 1985; Huff et al., 1989) suggest that GMC and neuron identity is mainly determined by NB identity and by lineage relationships. 'NB-identity' molecules are expressed in unique subsets of NBs; while 'sub-lineage-identity' molecules are expressed in NBs and their progeny only during a certain fraction of the lineage and generate differences between sister neurons (Cui and Doe, 1992; Doe, 1992). Finding new molecules with this cellular distribution is of special interest to uncover the mechanisms generating cellular diversity in the CNS.

Once postmitotic neurons start axonogenesis then epigenetic interactions with the surroundings are essential to direct their growth cones (Doe et al., 1985). In this context, molecules have been described that are expressed on the surface of

restricted subsets of axons. They could be part of a molecular code governing the formation of the multiple axonal pathways. 'Axonal-identity' molecules could be functioning as receptors for a specific guidance cue or as the signal for other axons that fasciculate with, or grow away from them. The fasciclins, described in both grasshopper and fly (Bastiani et al., 1987; Harrelson and Goodman, 1988; Patel et al., 1987; Kolodkin et al., 1992), are examples of axonal-identity molecules that possess a second level of restriction; they are expressed regionally only in portions of the axons defining certain pathways (e.g. commissural versus longitudinal). The existence of these axonal- and pathway-identity molecules is a prerequisite for the 'labeled pathways hypothesis' (Goodman et al., 1982), which predicts that axon fascicles differentially labeled by surface molecules are used for growth cone guidance. This role has been assayed in invertebrates for several surface proteins by antibody perturbation (Harrelson and Goodman, 1988; Seaver et al., 1991; Kolodkin et al., 1992; Wang et al., 1992) or genetic mutation (Elkins et al., 1990; Hedgecock et al., 1990; Grenningloh et al., 1991; McIntire et al., 1992). Moreover, such a restricted molecular code has also been invoked in the developing vertebrate nervous system (Dodd and Jessell, 1988; Bixby and Harris, 1991; Kuwada, 1992) supporting the idea that common mechanisms of guidance have been conserved during evolution.

Lazarillo is a highly glycosylated protein anchored by a glycosyl-phosphatidylinositol (GPI) tail to the plasma membrane of a subset of neurons in the nervous system of the grasshopper embryo. The deduced Lazarillo sequence reveals that it belongs to the lipocalin family, a group of proteins functioning as extracellular carriers of small hydrophobic

molecules (Ganfornina, Sánchez and Bastiani, 1995). In this work we report the developmental expression pattern and functional analysis of this new lipocalin. Its unique distribution suggests that Lazarillo is a sublineage- and axonal-identity molecule in the developing nervous system. Its presence in non-neuronal tissues suggests other functions more in agreement with the lipocalins found in invertebrates. To test its role in axon guidance we have used the monoclonal antibody (mAb) 10E6 to perturb the protein in cultured grasshopper embryos. Binding of the mAb to its epitope causes a subset of growth cones to be delayed and misrouted. We have centered our study on the altered behavior of an identified pair of commissural pioneer neurons. The cellular and molecular mechanisms by which a GPI-anchored membrane lipocalin may be guiding these commissural neurons are discussed.

MATERIAL AND METHODS

Grasshopper (*Schistocerca americana*) embryos were obtained from a colony maintained at the University of Utah. They were staged by percentage of embryonic development according to Bentley et al. (1979). The mAb 10E6 was generated by Carpenter and Bastiani (1990) against embryonic nervous tissue using a subtractive immunization method (Hockfield, 1987).

Immunocytochemistry and in situ hybridization

The antibody labeling protocol is a modified version of the procedures by Denburg et al. (1989). Embryos were removed from the egg case and dissected in Ringer's solution (4.6 mM Tes, pH 7.4, 150 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄) with 6 mg/ml of glycine, fixed in 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.2 and washed in PBS (20 mM potassium phosphate, pH 7.2, 150 mM NaCl). Embryos were incubated for 1 hour in blocking solution: 50 mM Tris, pH 7.2, 350 mM NaCl (50 mM TBS); 30 mg/ml bovine serum albumin (BSA fraction V, Sigma); 2% normal goat serum. mAb 10E6 was used as ascites fluid diluted 1:200 or hybridoma supernatant diluted 1:1 in blocking solution. Embryos were incubated with the primary antibody at 4°C for 8–12 hours, then washed with 50 mM TBS, blocked again and exposed to HRP-conjugated goat anti-mouse IgG (Jackson Immunoresearch) diluted 1:500 in blocking solution for 2 hours at room temperature, washed again with 50 mM TBS with a last change to 0.1 M TBS (0.1 M Tris, pH 7.2, 350 mM NaCl). Embryos were reacted with diaminobenzidine (1 mg/ml), glucose oxidase (3 mU/ml, type V) and β -D-glucose (2 mg/ml), washed in 0.1 M TBS and cleared in glycerol. Differential interference contrast microscopy (DIC) was used to analyze the preparations. Occasionally a secondary antibody conjugated to FITC or rhodamine was used and preparations were analyzed by standard fluorescence microscopy. To study the pattern of expression in grasshoppers ranging from 60% of development to 3rd instar adults, nerve cords were dissected and the standard protocol was followed using longer incubation times for antibodies (3–6 days for the primary and 18–24 hours for the secondary antibody) and adding 1% saponin to the blocking and antibody solutions. Adult nerve cord sections were obtained by embedding fixed tissue in OCT medium (Miles), sectioning at -18°C and fixing in 10% formaldehyde. Antibody labeling proceeded as above.

In situ hybridization procedures are detailed in Ganfornina, Sánchez and Bastiani (1995).

Antibody perturbation experiments

The mAb 10E6 used as a perturbing agent on cultured embryos was affinity purified from hybridoma supernatants using a Protein G Sepharose column (Pharmacia). The monovalent Fab fragments were prepared as described by the Immunopure Fab Preparation Kit

(Pierce). The concentration of antigen-binding sites was estimated taking into account absorbance at 280 nm, relative molecular mass, and number of antigen binding sites in each molecule.

Grasshopper embryos were dissected in medium consisting of: 50% Schneider's *Drosophila* Medium (Gibco-BRL), 49% Minimum Essential Medium (α medium, Gibco-BRL), 1% antibiotic-antimycotic solution (Sigma), 0.5 ng/ml juvenile hormone I and 0.3 ng/ml β -ecdysterone (Sigma). Each embryo was transferred to a HLA plate well (Nunc) containing 20 μ l of prewarmed medium and the experimental or control antibodies. Embryos were incubated at 33°C for 24 hours. Embryos used for each perturbation experiment were chosen from a single clutch and 4 embryos were fixed at the initiation of culture period ('t=0' embryos). After the culture period, embryos were washed in Ringer's solution and then fixed and labeled as described for immunocytochemistry. Embryos that were cultured with mAb or Fab 10E6 were subsequently exposed to a secondary antibody (HRP-conjugated goat anti-mouse IgG, from Jackson Immunoresearch). Control and t=0 embryos were labeled with mAb 10E6 and secondary antibody as described above. The mAb 10H11 (obtained in the same screening as mAb 10E6) and anti-HRP antibodies (Jackson Immunoresearch) were used in control experiments because both are nervous system specific and label the AcP cells.

To analyze the effects of the immunoperturbants used we chose the AcP cells of the S2 segment (see Results). A percentage of development was assigned to each cell according to Fig. 5F. Total growth achieved was estimated by subtracting the value of each control or experimental cell from the average of t=0 embryos. The mean growth and s.e.m. were calculated for the control and experimental groups of each experiment. To graphically represent the results of all experiments, they were grouped by type (A or B, see Results), antibody used (none, mAb 10H11, anti-HRP antibodies, mAb or Fab 10E6), and antigen binding site concentration. In each group of experiments the difference between experimental growth values and the mean value of control growth (growth cone delay) were calculated and its mean and s.e.m. were represented normalized to the mean growth obtained by control embryos in culture. The nonparametric test of Mann-Whitney was used to assess statistical significance of differences in growth. Altered AcP cells were scored when a major deviation from the normal pathway (Fig. 5) was observed. Cases were not scored when only filopodia extended along aberrant pathways. A χ^2 test was used to assess differences in frequency of abnormalities.

RESULTS

A general view of the Lazarillo expression pattern revealed by labeling with mAb 10E6 is outlined in Fig. 1. At 14% of development the embryos consist of an anterior enlarged portion, the presumptive head, and a smaller posterior primordium. As this primordium enlarges, gastrulation and segmentation occur sequentially in an anteroposterior direction. At 24% of development (Fig. 1A) segments are morphologically defined up to the second abdominal segment. The first pair of neurons labeled by mAb 10E6 appear in the head at 22% of development. Their axons will cross the midline by the primary commissural tract of the brain. Slightly later, a more lateral group of neurons is also labeled in the head. At 30% of development segmentation is complete and the embryo consists of a head, three subesophageal segments (S1-S3), three thoracic segments (T1-T3) and eleven abdominal segments (A1-A11). The ventromedial region of the ectodermal layer has been defined as the neurogenic region throughout the embryo and a stereotyped set of neuroblasts (NBs) differentiate in each segment. The neurons they produce will set up an orthogonal scaffold of axon tracts consisting of two longitudinal connec-

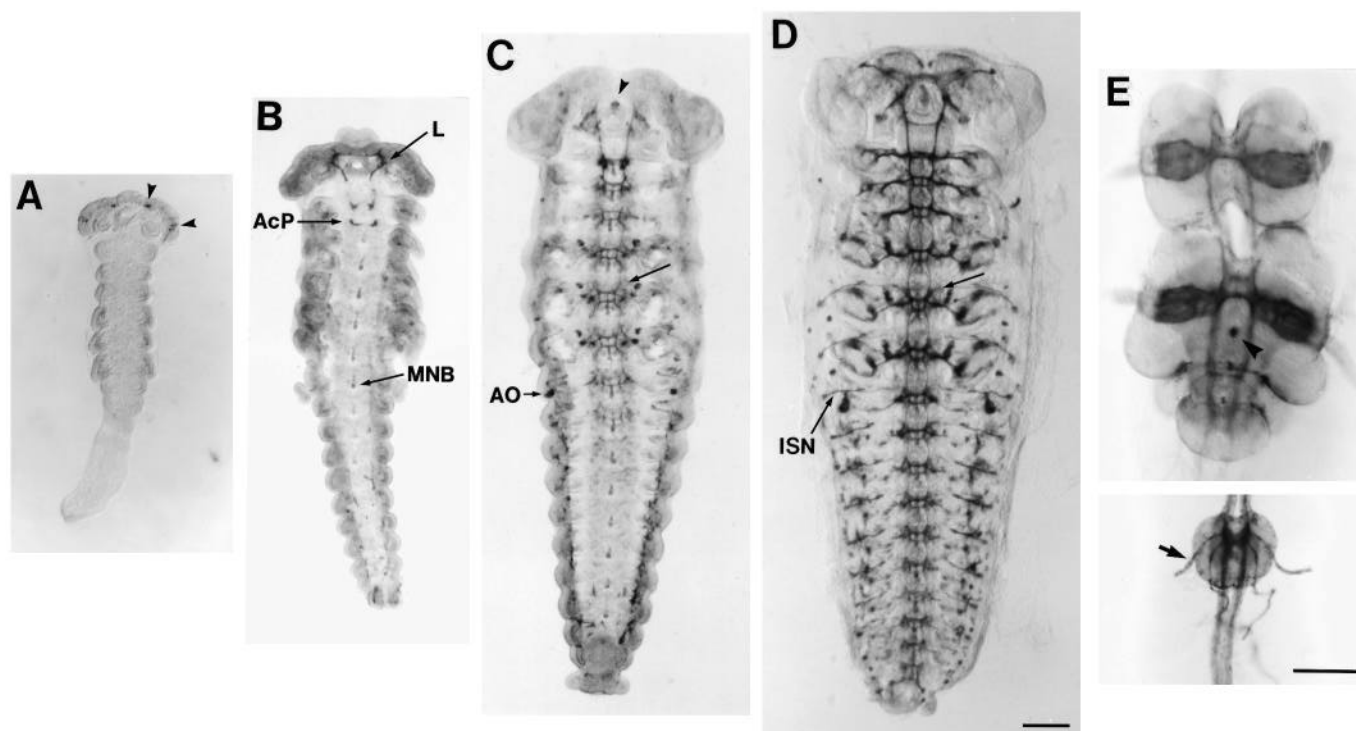


Fig. 1. Developmental expression pattern of Lazarillo in the grasshopper embryo. Lazarillo is visualized by immunocytochemistry with mAb 10E6. Whole embryos are viewed from the dorsal surface with DIC optics. Anterior is up. (A) At 24% of development a medial and a lateral group of cells in the developing brain (arrowheads), express Lazarillo on their surface. (B) At 32% of development, labeling appears on the primary commissure and longitudinal connectives (L) of the brain. The median neuroblast (MNB) and its progeny appear labeled at the midline of the more anterior segmental ganglia. Four cells in the subesophageal segments are intensely labeled with mAb 10E6. The pioneers of the anterior commissure (AcP neurons) are indicated by an arrow. (C) At 38% of development the first commissural fascicles appear in the most anterior segments (example shown with long arrow in T2 segment). Sensory neuronal clusters start to label in the lateral body wall. AO, auditory organ in A1 segment. The frontal ganglion of the enteric nervous system is also labelled (arrowhead) on the anterior margin of the foregut invagination. (D) At 45% of development, the complete neural expression pattern of Lazarillo is seen. Commissural and longitudinal fascicles are evident in the brain and ventral nerve cord (compare the T2 segment in C and D), and well developed sensory organs and nerves (ISN, intersegmental nerve) are present at the lateral body wall. (E) Ganglia of T2-A2 segments (upper panel) and A5 (lower panel) are shown at 80% of development. Commissural and longitudinal pathways are labeled as well as the peripheral nerves exiting the ganglia (arrow in lower panel). The MNB is labeled at this age (arrowhead in the T3 ganglion). Bars: A-D 300 μ m, E 150 μ m.

tives running between segments, two commissures linking both hemiganglia and a dorsal median fiber tract (MFT) that extends anteriorly along the midline. At 32% of development (Fig. 1B) 10E6 labeling appears in the brain commissure, in the longitudinal tracts connecting the brain with the segmental ganglia, in some brain NBs, in commissural neurons in S1 and S2 segments, and in MFT neurons up to the A2 segment. At these early stages other neurons that have extended their axons do not express Lazarillo. At 38% of development (Fig. 1C) the CNS scaffold has acquired more axonal tracts, some of which label with the mAb. Sensory neurons expressing Lazarillo are also differentiating in the body wall and appendages. At 45% of development (Fig. 1D) the axonal scaffold of the brain and segmental ganglia is completely outlined. Completion of the CNS will be carried out mostly by addition of new neurons which will follow preformed axonal fascicles. Sensory and motor axons travel along either the segmental nerve (SN) or the intersegmental nerve (ISN). These peripheral nerves are labeled by mAb 10E6. The enteric nervous system (ENS) that develops on the surface of the foregut invagination also expresses Lazarillo (Fig. 1C,D). Fig. 1E shows the ganglia T2,

T3-A3 (fused together), and A5 (lower panel) of an embryonic nerve cord at 80% of development. Lazarillo expression is maintained in the neuropilar region as well as in the longitudinal connectives and peripheral nerves up to hatching. The labeling in adult nerve cords appears restricted to certain groups of neurons and a ventral medial tract in the longitudinal connectives (Ganfornina, Sánchez and Bastiani, 1995, Fig. 1).

Identification of Lazarillo-expressing neurons in the grasshopper CNS

Lazarillo is localized on the membrane surface of the cell body, axon, growth cone and filopodia of a subset (5-10%) of the CNS neurons. At 32% of development (Fig. 2A) only a pair of cells that pioneer a fascicle in the anterior commissure and the MP4 and MP6 cells whose axons form the MFT are labeled. The median neuroblast (MNB) is also labeled. However, other cells that participate in forming the axonal scaffold do not express Lazarillo (open arrows). At 37% more neurons are added to the labeling pattern (Fig. 2B), such as a pair of longitudinal axons, a fascicle in the posterior commissure, and

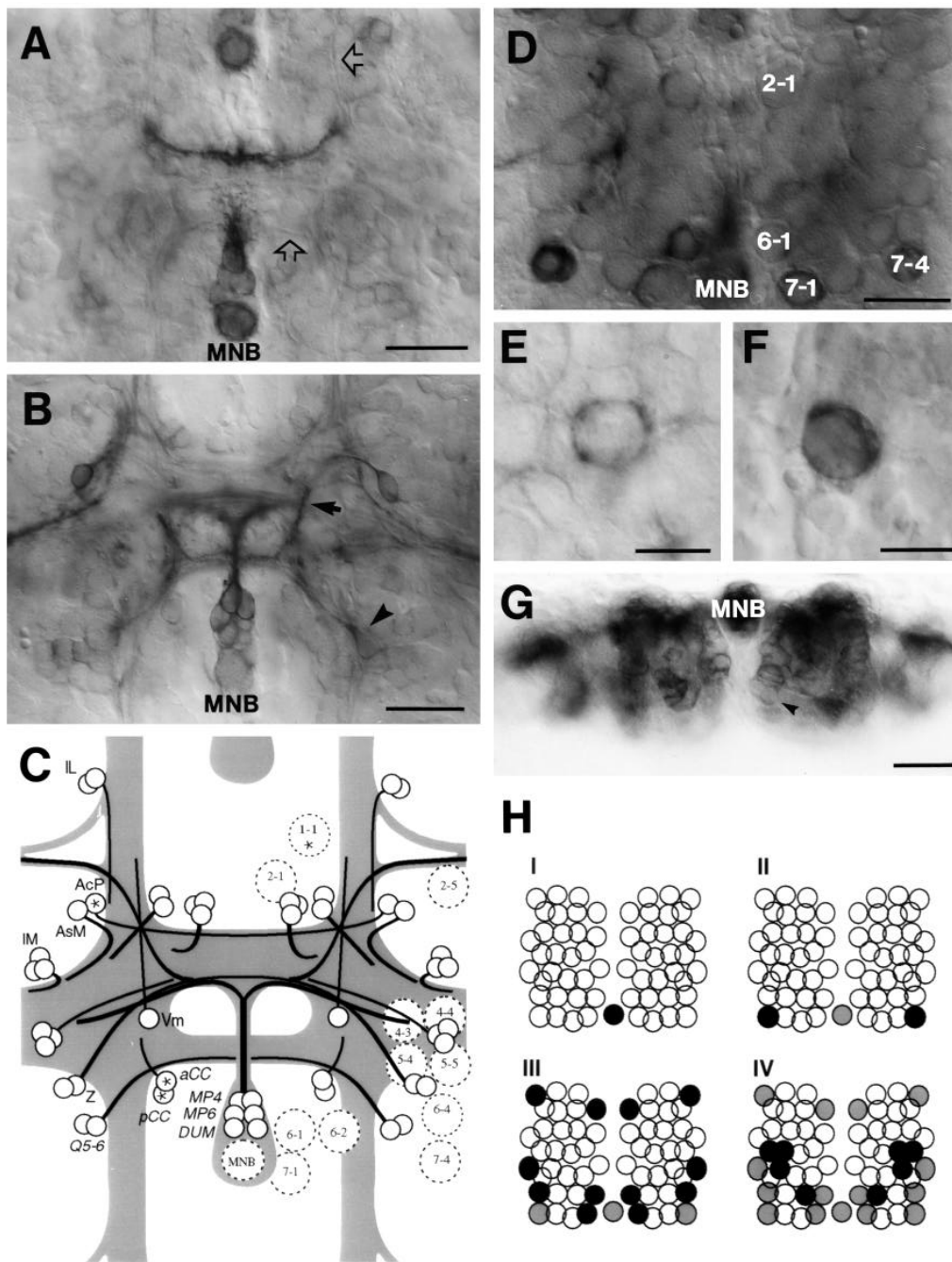


Fig. 2. The expression of Lazarillo in the CNS is restricted to a specific subset of neurons, NBs and GMCs in a precise temporal window. (A) The labeling at 32% of development in the metathoracic ganglion is seen on the neurons that pioneer the anterior commissure (AcP cells) and the MFT (MP4 and MP6 cells), as well as on the MNB. Refer to C for identity of neurons. Open arrows indicate unlabeled axons. (B) Later on (37%), more neurons express Lazarillo as they differentiate: for example the Q5 and Q6 cells (arrowhead) that cross the midline by the posterior commissure, or the Vm neurons that extends along a medial longitudinal fascicle (arrow). (C) Map of the 10E6-labeled neurons and NBs at 45% of embryonic development. The pattern of NBs labeled by mAb 10E6 is shown on the right hemisegment with dashed lines. Newly identified neurons are: anterior commissure pioneers (AcP), anterior segmental motoneurons (AsM), lateral motoneurons (IM), ventromedial tract neurons (Vm) that follow the vMP2 fascicle, the Z neurons that contribute to the posterior bundle of the anterior commissure, and the IL (lateral longitudinal neurons) that run along the A/P fascicle. Previously identified neurons (in italics) are: the corner cells (aCC and pCC), Q5 and Q6, MP4, MP6 and DUM (dorsal unpaired motoneurons). NBs and neurons noted with asterisks have segment-specific characteristics. NB 1-1 is labeled only in the S2 segment and the aCC and pCC cells only in the S1 segment. The intensity

of labeling of the AcP cells is much higher in S2 than in other segments. (D) A single focal plane of an A2 segment at 34% of development is shown as an example of NBs expressing Lazarillo. (E,F) The NBs themselves and not the surrounding sheath cells express Lazarillo as evidenced by the correlation between in situ hybridization with a Lazarillo RNA probe (E) and mAb labeling (F) of the MNB. (G) Caudal view of a metathoracic ganglion at 37% of development (dorsal is up). Families of cells derived from NBs 6-1 and 6-2 are seen on both sides of the midline. The arrowhead points to a NB 6-1 GMC. The expression of Lazarillo in NB 6-1 has started to fade. No neuroepithelial cells at the ventral surface of the ganglion show labeling. The MNB family is seen darkly labeled at the dorsal midline. (H) The mAb 10E6 labeling appears sequentially in NBs. The diagrams represent a thoracic ganglion from 26% (I) to 30% (IV) of development at approximate intervals of 1%. At each stage, newly appearing NBs are depicted in black, while previous ones are in gray. The order of appearance is as follows. I: MNB; II: NB 7-4; III: NBs 2-1, 2-5, 5-6, 6-1, 6-4, and 7-1; IV: NBs 4-3, 4-4, 5-4, and 6-2. Scale bars: A-D and G, 50 μ m; E and F, 20 μ m.

neurons that exit the CNS along the anterior edge of the SN. Fig. 2C shows a map of the neurons labeled by mAb 10E6. Some of them have been identified previously (Goodman et al., 1984), as the MP4 and MP6, which contribute to the MFT, or the Q5 and Q6 neurons, which emerge from the NB 7-4 and

send their axons by the posterior commissure (see Fig. 2B, arrowhead). Examples of newly identified cells are the two lateral neurons we have named Z cells, which cross the midline by a posterior fascicle of the anterior commissure, and the Vm cells, which send their growth cones slightly dorsally and then

anteriorly (Fig. 2B, arrow) following the vMP2 fascicle. Other newly identified neurons are the AcP neurons, pioneers of the first fascicle of the anterior commissure (Fig. 2A). A group of lateral motoneurons (IM) exit the CNS by the anterior border of the SN. Finally the IL cells are located lateral to the longitudinal connectives by the anterior branch of the ISN and their axons navigate posteriorly fasciculating with the A/P fascicle. The siblings of the AcP neurons, which we call anterior segmental motoneurons (AsM) pioneer the SN (running along its anterior border) and could correspond to the motoneurons 1v described by Whittington (1989). There are many known neurons that do not label with mAb 10E6 such as the Q1, Q2 and vMP2 cells, that pioneer the fascicles followed by the Q5-6 cells and the Vm cells respectively. The aCC and pCC are labeled only in the S1 segment.

Lazarillo is a NB-identity molecule expressed by a subset of NBs and GMCs in the CNS

mAb 10E6 reveals the presence of Lazarillo on a particular set of NBs. Several examples are shown in Fig. 2D (NBs 2-1, 6-1, 7-4 and 7-1) and the whole map is shown in Fig. 2C. GMCs closely associated with labeled NBs are also labeled (Fig. 2G). However, the neuroepithelial cells surrounding the NBs never express Lazarillo. The Lazarillo mRNA is present in the cytoplasm (Fig. 2E) and the protein is on the surface of the neuroblast (Fig. 2F). Lazarillo can therefore be considered a NB-identity molecule. Segment-specific differences are also observed, such as NB 1-1 only labeled in the S2 segment.

The NB expression of Lazarillo is not synchronous. The particular order of appearance of the labeling is depicted schematically in Fig. 2H. The MNB is the first labeled NB (at 26% development in the metathoracic segment, T3) and the last one to start expressing Lazarillo is NB 6-2 (at 30% development in T3). The onset of Lazarillo expression is not correlated with the order of NB birth described by Doe et al. (1985) and takes place once all NBs have differentiated from the neuroepithelium. NB expression of Lazarillo is also transitory. Labeling starts fading sequentially, starting with NB 7-4 and ending with the MNB, whose labeling is observed until 80% development (Fig. 1E; arrowhead). Because of the weak immunoreactivity of most NBs after 40% stage, it is difficult to establish when they cease the expression of Lazarillo. Moreover, some of them express Lazarillo intermittently, so we cannot rule out the possibility that NB labeling reappears later in development. These dynamic changes in expression are taking place while NBs are actively dividing to produce a portion of their progeny and therefore Lazarillo can also be considered a 'sublineage-identity' molecule (Cui and Doe, 1992).

In the cases where the lineage has been traced there is a clear correspondence between 10E6-positive neurons and the progenitor NB also being labeled. Examples are the Q5 and Q6 cells and NB 7-4, or the DUM neurons and the MNB, and the particular case of aCC and pCC cells in the S1 segment that are the progeny of NB 1-1 of segment S2. A subset of cerebral NBs is also labeled by mAb 10E6, but we have not attempted a detailed analysis of them.

Lazarillo is present in the peripheral and enteric nervous system

In the PNS only neurons, and not supportive non-neuronal cells, of sensory organs label with the mAb. The pattern is less

restricted than in the CNS; all described sensory neurons (Bentley and Keshishian, 1982; Meier et al., 1991) express Lazarillo. The labeled sensory groups that travel to the CNS through the ISN are shown in Fig. 3A. Sensory neurons of the SN are also labeled (not shown). The only unlabeled axon fascicle is the anterior branch of the ISN, or U fascicle, composed at this age of motoneuron axons exiting the CNS. The group termed IGp (lateral guidepost) cells is uniquely labeled by mAb 10E6. Meier et al. (1991) studied the grasshopper PNS in the body wall using anti-HRP antibodies as a general neuronal marker and they reported this particular group of cells (they called it ISO2) as being weakly or rarely labeled and only in T1-A1. A double labeling of segment A2 with mAb 10E6 detected with a rhodamine-conjugated secondary antibody and anti-HRP antibodies conjugated to FITC shows that anti-HRP antibodies do not label the IGp cells while mAb 10E6 does (Fig. 3B,C). The labeling of IGp cells is very intense in comparison to other sensory groups, and is present from T1 to A10 segments. IGp cells send their neurites toward the lateral dorsal body wall (dBw) cells before these begin axonogenesis (Fig. 3D). Later on, the dBw axons contact the IGp axons, fasciculate with them and navigate to the CNS. The IGp cells appear labeled at least until 60% of embryonic development, but we do not know the fate of this putative sensory organ.

The appendages of grasshopper embryos have been a model for studies on guidance of pioneer axons (reviewed by Bentley and O'Connor, 1992). The first sensory cells that send their axons to the CNS, the pioneer Ti1 neurons, follow a highly stereotyped pathway in which they encounter neuronal guidepost cells (Fe1, Tr1, and Cx1). Lazarillo is expressed by the sensory neurons of the developing legs. Fig. 3E shows a metathoracic leg at 32% of embryogenesis. Both the Ti1 and the guidepost cells are 10E6 positive, but with a darker labeling in the latter. All subsequent differentiating groups of sensory neurons are also labeled (see Figs 3, 6 in Ganfornina, Sánchez and Bastiani, 1995). Every sensory neuron is labeled in the antennae (Fig. 3F), labrum, mouth appendages and cerci.

Unlike in the CNS, Lazarillo is absent in the sensory organ mother cells (SOCs) in the PNS. Neurogenesis of sensory cells takes place in the periphery, close to the final location of the sensory organ. A SOC differentiates within the epithelium and divides symmetrically giving rise to a pair of neurons that delaminate into the lumen and start extending their axons along the inner surface of the epithelium. Fig. 3F shows two daughter neurons in an antenna before delamination from the epithelium.

A subpopulation of cells expressing Lazarillo are also observed in the outer layer of the foregut invagination where the ENS is developing (Fig. 1C,D). These cells will eventually form part of the frontal ganglion (not shown).

Expression of Lazarillo outside the nervous system

Lazarillo is not strictly specific of the nervous system. In the Malpighian tubules, a part of the insect excretory system, Lazarillo is present in the six cellular groups that initially differentiate from the proctodaeum epithelium at 45% of development (Fig. 4A). While the tubes are elongating the expression is restricted to a few cells at the tips (Fig. 4B). By 70% a new round of Malpighian tubule differentiation takes place; again Lazarillo appears at the tip of newly growing

tubules (not shown). It is intriguing that other genes have been related to selection of progenitor cells in both the nervous system and the developing tip cells of Malpighian tubules (reviewed by Campos-Ortega, 1994). At later stages (65% and older embryos) Lazarillo expression always correlates with a distended shape of the tubule tips. Chapman (1971) reported that Malpighian tubule tips appear swollen during periods of active transport of constituents from the haemolymph to the lumen of the tubules.

Other 10E6-labeled cells are distributed along the sides of the body wall (Fig. 4E). We could trace the origin of these polymorphic cells to a group of mesodermal cells at the tip of the invaginating proctodaeum (Fig. 4C,D). They eventually lose contact with it and migrate anteriorly. The presence of Lazarillo mRNA in these cells (Fig. 4C) suggests that they produce Lazarillo and are not just taking it up from the hemolymph. Three main cellular types have been described in insects that could fit the characteristics of these Lazarillo-positive cells: sessile haemocytes, nephrocytes, or the fat body cells. Future experiments will identify these cells and define their function.

The subesophageal body also labels with mAb 10E6 (Fig. 4F). This is an arched structure composed of loosely arranged nephrocytes arising from the lateral mesoderm of the S1 segment and functionally involved in excretion and synthesis of hemolymph components (Crossley, 1985).

The mAb 10E6 perturbs axon outgrowth and guidance

After a close examination of the identifiable cells that express Lazarillo, we chose the AcP cells in the S2 segment to analyze the effects of the mAb on axonal pathfinding. These cells project their growth cones toward the midline when no other axons labeled by 10E6 are present in the area, and show a conspicuous labeling on the growth cone and filopodia that allows us to assess precisely their behavior. We also examined other processes taking place during the culture period that was chosen to study the AcP cells. It is important to note that the AcP cells label much more intensely in the S2 than in other segments with both immunocytochemistry and *in situ* hybridization.

The axons of the AcP cells follow the stereotyped pathway shown in Fig. 5. At 29%, the AcP cell bodies are located slightly ventral to the dorsal basal lamina and extend randomly directed filopodia (Fig. 5A). The AcP growth cones extend toward the embryonic midline at 32% of development (Fig. 5B). They encounter the contralateral AcP growth cone and both axons fasciculate together at 34% of embryogenesis (Fig.

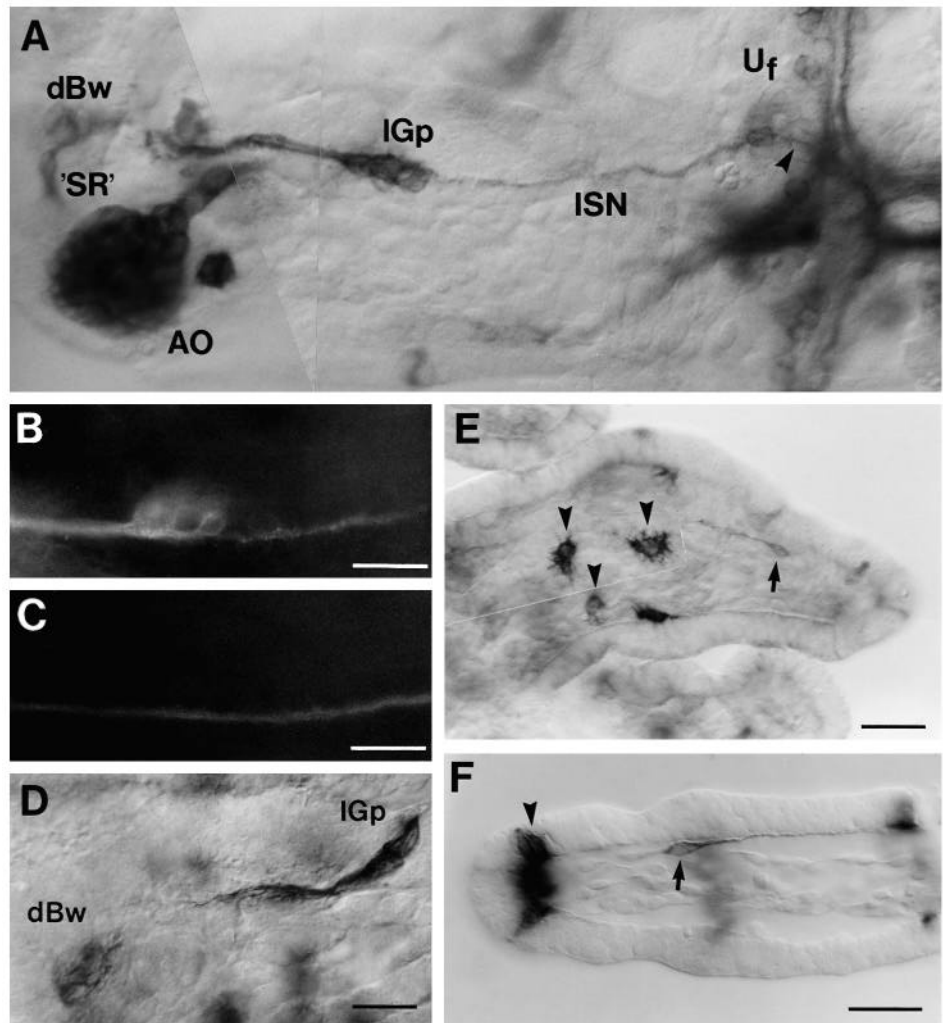


Fig. 3. Lazarillo is present on all sensory neurons in the PNS of the body wall and appendages. In A-D, CNS is on the right, and anterior is up. (A) Sensory neurons entering the CNS via the intersegmental nerve (ISN) are shown labeled by mAb 10E6. The anterior branch of the ISN, or U fascicle (U_f), is never labeled, while axons travelling by the posterior branch are labeled (arrowhead). dBw, dorsal body wall group; 'SR', wing hinge stretch receptor homologue in abdominal segments; IGp, lateral guidepost cells; AO, auditory organ. (B,C) A new neuronal group in the ISN (IGp cells) has been identified with mAb 10E6 to be present in all segments from T1 to A10. Anti-HRP antibodies, commonly used as a general neuronal marker, do not recognize these cells. A double labeling of the A2 segment is shown with mAb 10E6 evidenced with rhodamine-conjugated secondary antibodies (B) and anti-HRP antibodies directly tagged with FITC (C). (D) IGp cells send their neurites towards the more distal dBw cells before the latter start axonogenesis. The dBw axons follow the former as guideposts on their way to the CNS. (E) At the 32% stage the pioneer sensory neurons of the legs (Ti1, arrow) and the guidepost neurons (Fe1, Tr1, and Cx1, arrowheads) are labeled by mAb 10E6. Guidepost cells always label more intensely. Other neurons that are differentiating from the leg epithelium are seen out of focus. (F) Example of a sensory neuron in the antenna (arrow). The arrowhead points to two sibling cells that will become sensory neurons, which have not yet delaminated from the epithelium. Scale bars: A-C, 50 μ m; D, 25 μ m; E, 75 μ m; F, 40 μ m.

5C). During this period other commissural neurons start to follow the AcP axons, but they are not labeled by mAb 10E6 (not shown). The AcP growth cone reaches the contralateral cell body and then turns anteriorly at 36% of development, following the longitudinal vMP2 axon (open arrow, Fig. 5D), which is not labeled by mAb 10E6. The AcP axons can be considered followers along this pathway. They reach the posterior commissure of the S1 segment at 39% of development (Fig. 5E). All the stages are schematically drawn in Fig. 5F. We defined two types of antibody perturbation experiment based on culture periods that give us the greatest temporal resolution to analyze the AcP pathway. Type A experiments were started at 29-32% of development, while type B experiments were started at 33-35%.

Two effects were observed in embryos cultured with mAb or Fab 10E6. (1) The position of AcP growth cones was delayed with respect to control embryos. Representative examples are shown in Fig. 6. In the type A control experiment (Con) the AcP cells of the S2 and A1 segments reach the midline at 33% of development. In experimental embryos (Exp) the AcP cells of S2 showed a considerable delay while AcP cells in all other segments of the same embryo grow normally. Segment A1 was chosen for comparison because it has a developmental stage similar to S2 (Patel et al., 1989). The type B experiment shown in Fig. 6 was started at 33% of development. These experimental AcP cells remained at a stage equivalent to 33%, while control cells reach the next anterior segment, a stage equivalent to 38%. This represents a 100% delay compared to control growth (see Materials and Methods). Although the degree of delay varies, experimental AcP growth cones are frequently found stalled at the contralateral cell body in type B experiments. The effects are described as a delay in position because our experimental assay does not allow us to distinguish among a stop in growth, a lower rate of growth or normal growth followed by axon retraction.

In both types of experiment, embryos cultured in the presence of 8.8 μM mAb 10E6 showed a delay of approximately 35% compared to the growth attained in controls (see Table 1 and Fig. 6). It is important to note the absence of delay in some experimental AcP cells, which we

propose could be explained by a partial blocking of Lazarillo function by mAb 10E6. No differences were observed in AcP cells in other segments. The delay in both types of experiment increases with mAb concentration and reaches a saturation level as predicted for a specific interaction. Statistically significant differences in growth are observed at 0.88 μM and higher. Experiments with 8.8 μM of Fab antigen binding sites result in similar delays in growth in both types of experiment, ruling out an artifactual cross linking of Lazarillo molecules by the bivalent mAb. Other antibodies that label the AcP cells (see Materials and Methods) were used to assess whether other IgGs can cause the observed delays. Neither the mAb 10H11, nor the anti-HRP antibodies produced a significant delay. Data gathered from 52 independent experiments, including 369 control and 261 experimental embryos, are shown in Table 1.

(2) The AcP growth cones are misrouted during the culture period. In type A experiments (Fig. 7A-D), a significant propor-

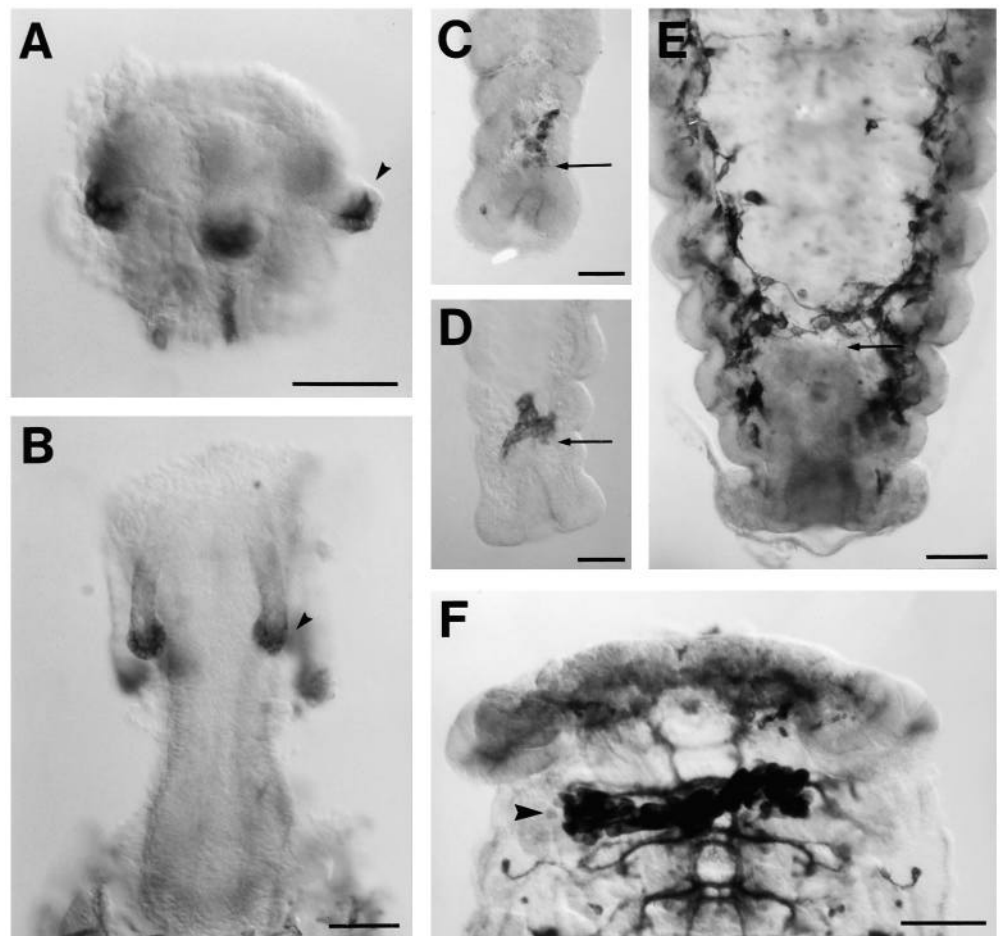


Fig. 4. Lazarillo is also expressed outside the nervous system. Embryos are viewed from the dorsal surface with DIC optics. Anterior is up. (A) The Malpighian tubules express Lazarillo as soon as they start invaginating from the proctodaeum into the body cavity (45% of development). Arrowhead points to one of the six groups of cells. (B) Labeled cells (arrowhead) are restricted to the slightly swollen tips of the growing tubules (55% embryo). (C,D) Amoebic-shaped cells expressing Lazarillo originate at the tip of the proctodaeum. (C) RNA in situ hybridization of a 29% embryo, (D) mAb labeling of a 32% embryo. (E) Lazarillo expression is maintained while these mesodermal cells are differentiating and migrating anteriorly along the sides of the abdominal body wall (40% embryo labeled with mAb). Arrow points the anterior edge of the proctodaeum in C-E. (F) The cells composing the suboesophageal body (arrowhead) are darkly stained with mAb 10E6 (45% embryo). Scale bars: A-E, 100 μm ; F, 200 μm .

tion of AcP cells cultured in the presence of mAb or Fab 10E6 showed an anomalous axon growth directed anteriorly toward the next S1 segment (53 of 332 cells in experimental embryos; 8 of 470 cells in controls; Fig. 7I). Axon extension in lateral or posterior directions was never observed. The AcP growth cones in type B experiments follow their normal pathway. However, abnormal anterior branches arising from the commissural portion of the axon were seen in embryos cultured with mAb or Fab 10E6 (Fig. 7E,I; 16 of 190 cells in experimental embryos; 4 of 267 cells in controls).

Defasciculation of AcP axons along the commissural pathway was never observed in either type of experiment. Profuse filopodia were observed in experimental embryos (Fig. 7F,G) but they were not scored. Interestingly, some control filopodia explore the anomalous pathways followed in experimental conditions (Fig. 7H), but they never extend to posterior and lateral areas, suggesting that in control embryos the anteromedial region is surveyed but not selected for growth.

Although not extensively analyzed, sensory axons normally growing along the posterior branch of the ISN are often delayed and have altered anterior orientations when cultured with mAb 10E6. However, other axonal pathways labeled by 10E6, such as the AcP neurons in other segments (Fig. 6), their sibling neurons (AsM) that pioneer the SN, the Ti1 neurons of the leg, and the MFT were normal. We have not, however, assayed all other labeled neurons at this time. Several longitudinal and commissural fascicles unlabeled by mAb 10E6 also appear correctly formed during the chosen culture periods, ruling out a general disruption of the axonal scaffold. The effects caused by the presence of mAb 10E6 or Fab fragments were evenly scattered throughout experiments, suggesting that no particular clutches of eggs were more susceptible to perturbation.

Thus, blocking the epitope recognized by mAb 10E6 causes the S2 AcP growth cones to delay, and often dramatically change their direction of growth. These results suggest that Lazarillo is involved in the outgrowth and guidance of specific growth cones in the nervous system of the grasshopper embryo.

DISCUSSION

Lazarillo is expressed throughout embryogenesis, postembryonic development and adulthood. We have focused

on its physiological role during embryogenesis. Although no other molecule has been described with the same tissue distribution as Lazarillo, a close resemblance exists with DSS-8, a cell surface protein in the cockroach embryo (Denburg et al., 1989). However, its localization in other tissues, as well as biochemical properties (Wang et al., 1992), weaken the possibility of homology between these two proteins.

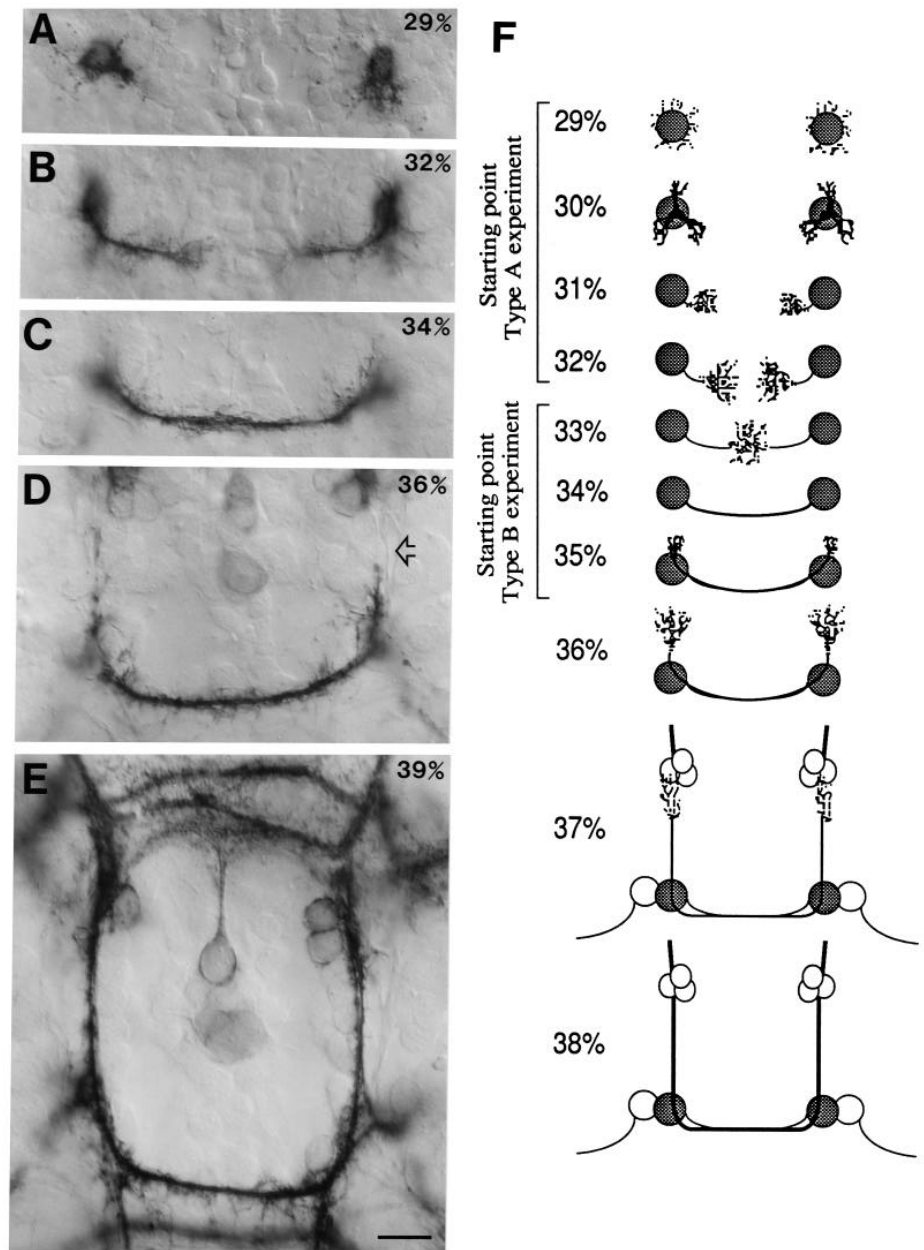


Fig. 5. The growing axons of the anterior commissure pioneer (AcP) cells follow a stereotyped pathway. (A) At 29% of development the AcP cells start producing filopodial extensions without any preferential direction. (B) At 32% of development the growth cone extends toward the midline. (C) At 34% of development the two AcP axons have crossed the midline and fasciculated with each other. (D) At 36% of development, after encountering the contralateral cell body, they extend anteriorly following the unlabeled vMP2 axon (open arrow). (E) At 39% of development the AcP axons have reached the posterior commissure of the next anterior segment (S1). Scale bar, 25 μ m. (F) Staging of AcP cells by 1% intervals. Two types of antibody perturbation experiment were conducted. Type A experiments started at 29-32% of development. Type B experiments started at 33-35% of development.

Table 1. Average delay values and total number of observations in each group of experiments

Antigen binding site concentration (μM)	0.176		0.88		1.76		8.88		17.6	
	n	delay(%)	n	delay(%)	n	delay(%)	n	delay(%)	n	delay(%)
TYPE A:										
Control	10	0±3.1	96	0±3.5	48	0±6.6	200	0±4.7	54	0±7.1
mAb 10E6	12	2.1±11.3	94	25.3±4.4*	50	33.3±5.1*	38	35.1±5.9*	54	38.9±5.8*
Fab 10E6							96	43.1±4.6*		
mAb 10H11							38	2.8±2.3		
αHRP serum							24	-3.1±2.7		
TYPE B:										
Control	12	0±8.8	36	0±5.5	48	0±4.1	102	0±4.1	26	0±6.3
mAb 10E6	12	-3.8±8.5	40	24.5±7.7**	48	37.3±6.1*	46	34.9±6.0*	26	25.7±7.3**
Fab 10E6							30	37.9±6.6**		
mAb 10H11							43	7.8±3.3		

Data expressed in mean±s.e.m.

Asterisks mean that the difference from the control was statistically significant (Mann-Whitney U test, **P*<0.005, ***P*<0.05).

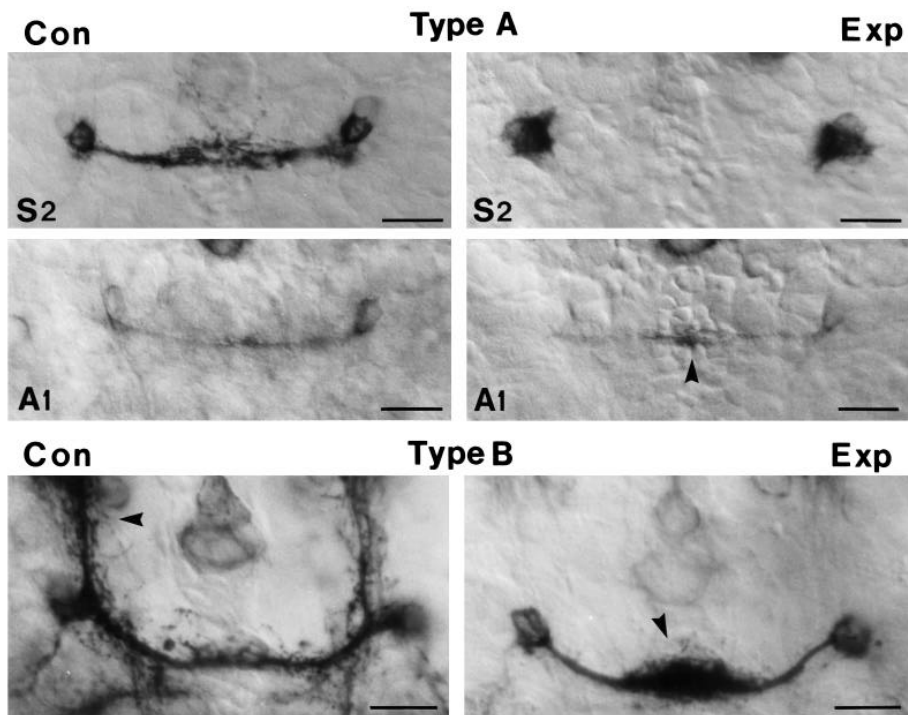
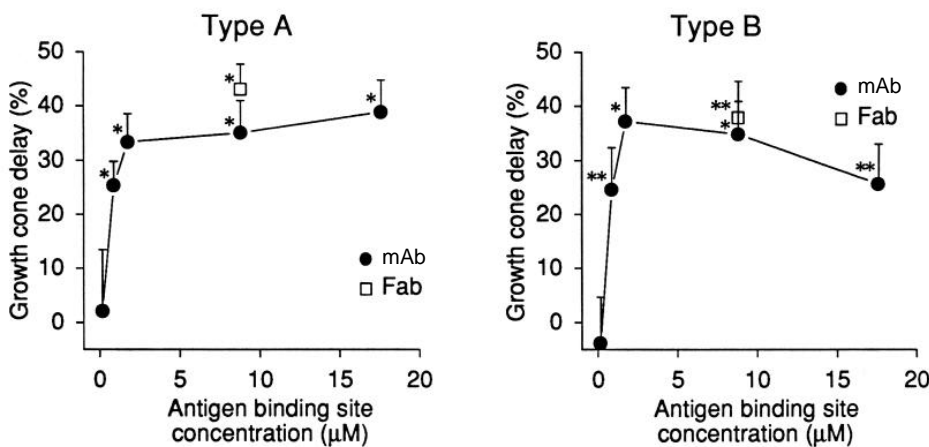


Fig. 6. The AcP axons are delayed in a dose dependent way when embryos are cultured in the presence of mAb 10E6 or its Fab fragments. In a Type A experiment, started at the 29% stage, the AcP cells are shown in the S2 and A1 segments of an embryo cultured for 24 hours in control conditions (Con) and another cultured with 8.8 μM of mAb 10E6 (Exp). A segment-specific greater intensity of labeling is observed in S2 where a clear arrest is seen in the experimental embryo. The AcP cells in control as well as in the experimental A1 segment have reached the midline (arrowhead). The type B experiment shown, started at 33% of development. In the control embryo the AcP growth cones have completed their navigation to the S1 segment (arrowhead in Con). In the experimental embryo, cultured with 8.8 μM of mAb 10E6, cells are still at the midline and their growth cones send abundant filopodia (arrowhead in Exp). Growth cone delay is plotted versus antigen binding site concentration in both types of experiment. Each cell was staged according to Fig. 5F, and the growth in culture estimated by subtracting the average stage of the t=0 embryos. Delay was defined as the difference between each experimental growth value and the average growth of the control embryos in a given experiment. The average delay in each group of experiments with the same concentration of immunoperturbant is normalized to the average growth attained in the control cultures. Error bars represent s.e.m. Asterisks indicate a statistically significant difference from the control (Mann-Whitney U test, **P*<0.005, ***P*<0.05). Scale bars: Type A, 30 μm; Type B, 25 μm.



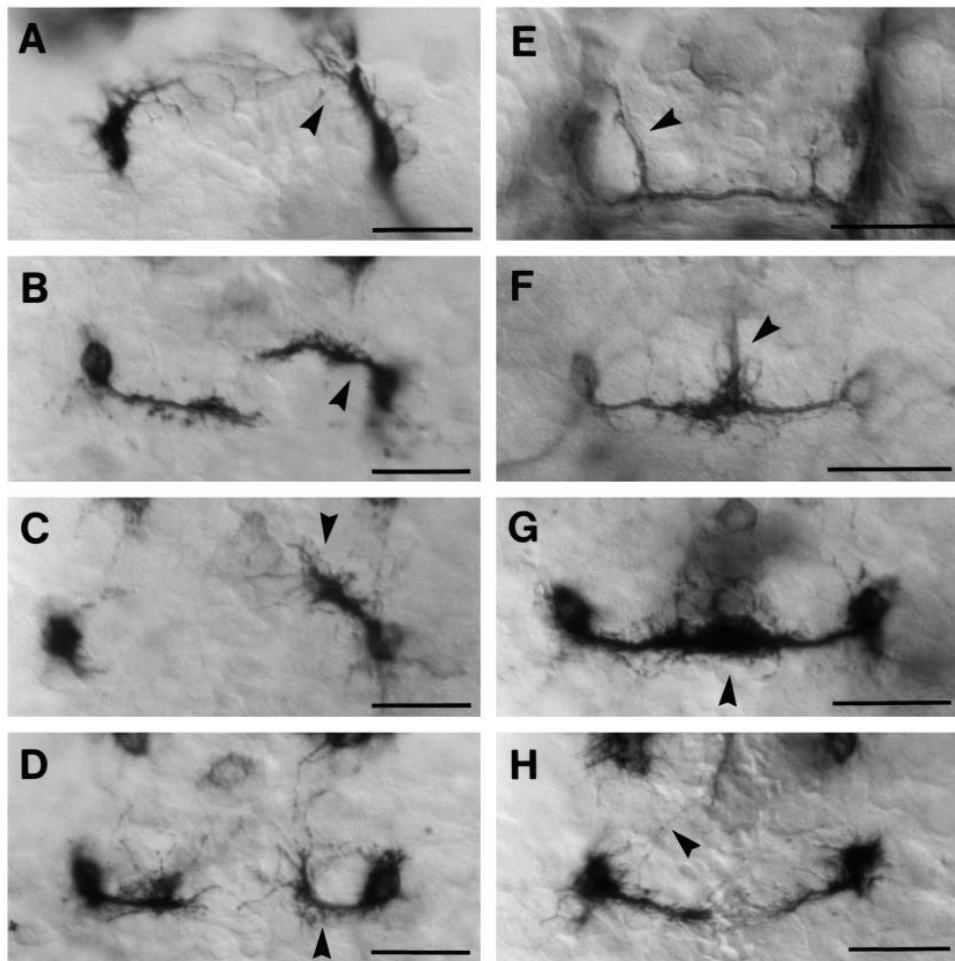
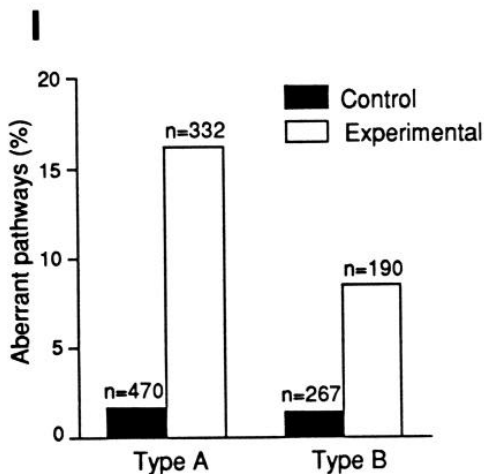


Fig. 7. The AcP axons of embryos cultured in the presence of mAb 10E6 or its Fab fragments choose aberrant pathways or extend aberrant branches. (A-D) Examples of anomalies found in type A experiments. Aberrant growth is always directed anteriorly with respect to the AcP cell body. (A) In this embryo both AcP axons grew straight anteriorly though long filopodia from the growth cone on the right (arrowhead) are also seen directed to the midline. Culture started at 29% of development. (B) While the cell on the left behaved normally in this embryo, the cell on the right sent its growth cone first anteriorly and then (arrowhead) turned to the midline. Culture started at 30% of development. (C) The growth cone on the right (arrowhead) is pointing to the midline but with an anomalous diagonal trajectory. The cell on the left is clearly delayed. Culture started at 29% of development. (D) When the experiment started after the axons are



already oriented toward the midline, the presence of the antibody causes sharp changes in direction. Arrowhead points to the position of the growth cone at the beginning of the culture period (32%). (E) In type B experiments, branches parallel to the normal longitudinal pathway often appear (arrowhead) always directed anteriorly. (F,G) Examples of filopodial anomalies not scored but frequently observed in experimental embryos. Delayed AcP growth cones send long filopodia anteriorly along the midline (arrowhead in F) or show profuse filopodial extensions at the point of contact with the contralateral growth cone (arrowhead in G). Embryos were cultured with 0.88 μM (F,G), 1.76 μM (D), 8.8 μM (A,E) of mAb and 8.8 μM (B,C) of Fab. (H) The aberrant pathways observed in the presence of 10E6 are normally explored by filopodia (arrowhead) in control conditions. (I) The percentage of experimental cells with anomalies was significantly different from the control population in both types of experiment (χ^2 test, $P < 0.005$). Total number of observations in each group is noted. The embryos cultured with mAb 10E6 (from 0.88 μM to 17.6 μM) and with 8.8 μM of 10E6 Fab are combined in the experimental category. Embryos cultured with no antibody, 10H11 mAb or anti-HRP polyclonal are combined in the control category. Bars: 50 μm .

Lazarillo is present in non-neuronal tissues, such as Malpighian tubules and the subesophageal body, both of which are involved in excretion. Since Lazarillo is a lipocalin, it could be capturing hydrophobic metabolites from the hemolymph to be internalized and stored (in the case of subesophageal body) or actively transported through the cell to the lumen of Malpighian tubules. This role would be in agreement with the functions of the lipocalins most related to Lazarillo, porphyrin- and retinol-binding proteins, with the interesting difference

that Lazarillo is a membrane bound lipocalin (Ganfornina, Sánchez and Bastiani, 1995). In contrast, the possibility that Lazarillo is involved in lipid transport and metabolism in the embryonic nervous system is unlikely, since its restricted pattern of expression would support the existence of a neuronal subset-specific regulation of lipid metabolism.

In the CNS, Lazarillo is first expressed by the NBs and GMCs in the brain and segmental ganglia, always after the appearance of the whole array of NBs, ruling out a role in NBs

differentiation. However, the restriction to approximately 40% of the NBs in a segmental ganglion suggests Lazarillo is a NB-identity molecule. No exact correlation is found between the subset of NBs labeled by 10E6 and the NB-identity molecules reported so far (Doe, 1992; Doe and Technau, 1993). Identified NBs and GMCs express Lazarillo during a particular time window and there are also examples of intermittent expression. These features suggest that Lazarillo belongs to the group of NB-identity molecules that specify the GMC identity in a sub-lineage-specific manner. The different cell types (NBs, GMCs and postmitotic neurons) that are involved in neuronal differentiation express a number of proteins (Doe, 1992; Doe and Technau, 1993) that appear distributed in different cellular compartments: nuclear proteins, which regulate gene expression, extracellularly secreted proteins, which might function as diffusible signals, and membrane bound molecules. Lazarillo, a membrane protein, could be acting as a receptor for morphogenetic signals participating in neuronal differentiation and determination. mAb 10E6 can be considered a sub-lineage-specific marker and will be a useful tool to ascertain new cell lineages, an essential requirement to understand the mechanisms generating cellular diversity in the nervous system.

An intriguing finding in the PNS is that Lazarillo is absent from the SOC. As every neuron arises by symmetrical division from its own SOC, neuronal sublineages need not to be formed during neurogenesis in the PNS. Therefore, sublineage-identity molecules might not be necessary. Likewise, once a cluster of sensory neurons is formed the emerging nerve shows no distinctive fascicle subdivisions. This could explain why Lazarillo expression is not restricted to subsets of sensory neurons. A particularly interesting sensory cluster associated with the ISN is labeled by mAb 10E6. This group we have called IGp, has been missed in other studies where anti-HRP antibodies, which recognizes a complex carbohydrate epitope (Snow et al., 1987), were used. The lack of correspondence between anti-HRP and 10E6 labeling suggests a different carbohydrate moiety in Lazarillo. Since the IGp cells send their neurites toward the periphery before any other axon travels the ISN, we propose a guidepost role for them. This contradicts the proposal that dBw cells pioneer the peripheral part of the ISN without help from any neuronal guidepost cell (Meier et al., 1991).

The sublineage constraint observed in the NB array expressing Lazarillo is in agreement with the restricted pattern of neurons and axonal bundles labeled by 10E6. No correlation has been detected though with a particular mature cellular type (motoneurons, interneurons and sensory neurons are labeled) or with cells bearing functional significance during development (fascicle pioneers and followers, neurons undergoing programmed cell death or not, all express Lazarillo). Nevertheless, we have not assayed all other possibilities e.g., a common neurotransmitter expression in the sublineages labeled by 10E6. There is, however, a striking example of a correlation with the pathway a certain neuron takes. The aCC, segmental motoneurons that exit the CNS by the anterior branch of the ISN, do not express Lazarillo in thoracic or abdominal segments, but they appear darkly labeled by 10E6 in the subesophageal segment S1 (Fig. 5E), where the ISN is absent and those motoneurons follow a different pathway.

The presence of Lazarillo on growth cones and filopodia,

and its restriction to particular axon fascicles during the processes of axonogenesis, fascicle formation and pathway selection, suggest a role in pathfinding. We tested this hypothesis by perturbing the molecular interactions of Lazarillo with mAb 10E6 in cultured grasshopper embryos. A pair of cells that pioneer the first fascicle in the anterior commissure (AcP cells) were chosen for this study and two kinds of perturbation were observed: a delay of the AcP growth cones and an altered trajectory of growth. Nevertheless, a remarkable absence of commissural defects occurs in the AcP cells of body segments other than S2, which correlates with segmental quantitative differences in the level of Lazarillo expression as revealed by immunocytochemistry and in situ hybridization. This could be explained either by proposing the existence of redundant guidance cues and a segment-specific role for Lazarillo in guiding commissural axons, or an only partial perturbation effect by mAb 10E6.

The AcP growth cone emerges from the cell body and invariably grows toward the midline. It is currently believed that guidance of commissural neurons relies on specialized midline cells, the floor plate cells in vertebrates (Tessier-Lavigne et al., 1988; Hatta, 1992) and the midline glial cells in invertebrates (Menne and Klämbt, 1994). There is evidence for both attractive and repulsive guidance cues provided by the midline, possibly in the form of soluble morphogen gradients, that determine commissural versus ipsilateral growth cone trajectories (reviewed by Goodman and Shatz, 1993; Goodman, 1994; Baier and Bonhoeffer, 1994). In those cases where the AcP growth cones are perturbed by mAb 10E6 they often stop growing toward the midline, and in a significant number of cases they turn and always grow in an anterior direction. As mentioned above, one could propose that Lazarillo participates in the lipid metabolism of the cell and, in this case, is necessary for the rapid expansion of membrane associated with axonogenesis. Nevertheless, many AcP axons were able to extend comparable distances along aberrant pathways, and we propose that the delay is a consequence of altering a specific signaling mechanism for directional guidance. The invariant anterior extension of abnormal growth cones is a strong indication for the existence of an anterior guidance signal.

The AcP growth cone normally encounters and fasciculates with its contralateral homologue at the midline. This contact with its homologue may allow the AcP growth cone to extend away from the peak of a midline morphogen. A similar mechanism in which cell-cell interactions are dominant to morphogen gradients has been proposed to account for the behavior of other pioneer neurons in the grasshopper embryo, such as the Q1 neurons in the CNS (Myers and Bastiani, 1993 and unpublished observations), and the Ti1 neurons in the limb bud (Bentley and Caudy, 1983; Caudy and Bentley, 1986). Defasciculation between the two AcP axons was never observed in the mAb blocking experiments, indicating that Lazarillo is not involved in this selective axon-axon recognition. However, although fasciculation with its homologue is normal there are many anomalous branches that extend anteriorly towards the segment border. There is strong experimental support for a role of boundary regions, such as the segment border, in axon guidance (Wilson et al., 1993).

Next, the AcP axon leaves its homologue to turn and grow anteriorly along the vMP2 fascicle. Commissural neurons must often initially ignore an ipsilateral target, but later specifically

recognize that same target on the contralateral side after traversing the midline. Experimental observations indeed suggest that axons traversing the midline can change their expression of cell surface proteins (Bastiani et al, 1987; Dodd and Jessell, 1988) and therefore acquire new pathfinding abilities (Rothberg et al., 1990; Campbell and Peterson, 1993). Immunoperturbed AcP growth cones are often delayed and found stalled at the contralateral cell body, suggesting that recognition of the vMP2 axon is impaired.

Although other hypotheses are formally possible, such as those derived from considering mAb 10E6 as an agonist of Lazarillo function, we propose the following model to account for commissure formation in the grasshopper embryo based on the behavior of the perturbed AcP growth cones. The AcP cells respond to segment wide mediolateral and anteroposterior morphogen gradients, possibly originating at the midline and segment border respectively. We propose that Lazarillo is the receptor for a midline morphogen. Blocking Lazarillo function with mAb 10E6 before the AcP cells reach the midline would prevent growth toward the midline and allow the growth cone to respond to an anterior morphogen. Once at the midline, specific cell-cell interactions would be dominant to the global gradients, allowing the AcP growth cone to extend away from the midline by fasciculating on its contralateral homologue. When the perturbed AcP growth cone reaches the contralateral side it is not able to recognize vMP2. We propose that altering the reception of the midline signal by perturbing Lazarillo would prevent the switch in the specificity of the AcP growth cone from its homologue to the vMP2 fascicle. In contrast, the response to the anterior signal seems to be independent of the midline signal, since the immunoperturbed AcP cell extends abnormal processes both before and after crossing the midline.

In this model the interplay between the anterior and midline morphogens determines the polarity of growth, and localized cell surface cues determine the precise position of pathways. The early mediolateral and anteroposterior segment gradients would account for the behavior of pioneering growth cones. The vast majority of later developing neurons would make use of substrate bound cues on individual fascicles for their pathway choices, but may still rely on these global gradients to define their polarity of growth. Lazarillo would be directly involved in the reception of the midline morphogen and indirectly in the switch in specificity for subsequent pathway choices. The identity of the proposed morphogens and the ligand that the lipocalin Lazarillo can bind is currently unknown, but the phylogenetic analysis of Lazarillo sequence suggests heme metabolites and retinoids as strong candidates (Ganfornina, Sanchez and Bastiani, 1995). In this context, it will also be important to test the binding of Lazarillo to soluble proteins secreted by midline structures such as the *slit* gene product (Rothberg et al., 1990) or the recently discovered members of the netrin family of outgrowth-promoting and axon guidance proteins (Serafini et al., 1994; Kennedy et al., 1994). Future experiments will test the predictions of this model.

The results presented in this paper suggest that Lazarillo is directly involved in the guidance of developing axons along their correct pathways. A multifunctional role of Lazarillo is also inferred from its complex expression pattern; it could also be involved in the specification of NBs and neuronal identities as well as in the development and function of the excretory

system of insects. A better understanding of its functional roles will be of interest to formulate new hypotheses about the cellular and molecular mechanisms governing the processes of neuronal identity determination and axon guidance.

We are grateful to the members of our laboratory and of the Biology Department for support and critical reading of the manuscript; C. Goodman for advice, and E. Carpenter for generating mAb 10E6. This work was supported by NIH grant to M. J. B. (NS25387). M. D. G. holds a fellowship from the Fulbright/MEC of Spain, and D. S. is the recipient of a NIH postdoctoral fellowship (1F05TW04686-01).

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(Accepted 30 September 1994)