TARGETING OF SENSORY NEURONS DURING LEECH EMBRYOGENESIS MEDIATED BY CARBOHYDRATE INTERACTIONS

By

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ABSTRACT

TARGETING OF SENSORY NEURONS DURING LEECH EMBRYOGENESIS MEDIATED BY CARBOHYDRATE INTERACTIONS

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This thesis described the molecular mechanisms regulating the targeting of sensory neurons in the intact nervous system of the embryonic leech, *Hirudo medicinalis* . I first determined the normal differentiation of sensory afferents in vivo. Then I developed an in vitro embryo culture system that permits the normal differentiation of sensory afferent projections within the CNS neuropil. Using this culture system, I studied the kinetic process of the molecular perturbations of axonal projection. I also investigated the functions of two carbohydrate markers that distinguish between two different subsets of these sensory afferents.

As sensory afferents integrate into neuronal networks, the pattern of their axonal projections demonstrates two characteristic changes. First, afferents defasciculate upon entry into the CNS after they projected as tight bundles through peripheral nerves. This defasciculation is mediated by a constitutive mannose-containing epitope on their cell surface (Zipser, et al., 1989). I now demonstrated that the rate at which defasciculated axons collapse back into larger axon bundles can be modeled by a first order decay reaction. Second, two days after entering the synaptic neuropil, sensory afferent neurons begin to assemble into stereotypic target regions. I found that the assembly of the previously diffuse axonal projection into target regions correlates with the onset of expression of new surface carbohydrate markers. Previously it was shown, using adult leeches, that these developmentally regulated carbohydrate markers identify two subsets of sensory afferents with different sensory modalities (Zipser et al., 1994) . Characterizing these two subset carbohydrate markers I found that one of them contains galactose, whereas the other one contains both glucose and galactose. Using molecular perturbation with Fab fragments, neoglycoproteins and enzymes, I demonstrated that each

subset carbohydrate marker mediates the attachment of its respective subsets to a unique target region of the CNS. Thus, the mannose-containing marker shared by all afferents and the hybrid/complex markers that define functional subsets of these same sensory afferents collaborate in the targeting of two axonal subsets during the development of neuronal connectivity.

To my wife, Jianong, and my daughter, Fangwen.

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α–Gal	α–Galactosidase
α–Glc	α–Glucosidase
β–Gal	β–Galactosidase
β–glc	β–Glucosidase
β–GlcNAc	β–N-Acetylglucosaminidase
α–Man	α–Mannosidase
Cell	Cellulase
CNS	Central nervous system
CE0	Carbohydrate epitope 0, which contains mannose, is shared by the fullset of sensory afferent neurons. This epitope is recognized by eight different mAbs, among them Lan 3-2
CE1	Carbohydrate epitope 1, which contains α–galactose, is expressed by a large subset of sensory afferents neuron. This epitope is recognized by mAb Laz 2-369
CE2	Carbohydrate epitope 2, which contains both glucose and α–galactose, is expressed by a medium size subset of sensory afferent neurons. This epitope is recognized by four different mAbs among them Laz 7-79

CE3	Carbohydrate epitope 3, which contains β–galactose, is expressed by a small subset of sensory afferent neurons. This epitope is recognized by mAb Laz 141
DAB	3,3'-diaminobenzidine
EDTA	ethylenediaminetetraacetic acid
Fab	binding fragment of antibody generated by papain digestion
Fuc-BSA	Albumin, Bovine-fucosylamide
Gal-BSA	Albumin, Bovine-galactosamide
Glc-BSA	Albumin, Bovine-glucosamide
GlcNac-BSA	Albumin, Bovine-p-Aminophnyl-N-Acetyl-β–D-glucosaminide
HRP	horseradish peroxidase
kD	kilodaltons
mAb	monoclonal antibody
mAb Lan 3-2	recognizes CE0, the mannose-containing epitope expressed by all sensory afferents
mAb Laz 3-369	recognizes CE1, the α–galactose-containing epitope expressed by a large subset of sensory afferents
mAb Laz 7-79	recognizes CE2, the glucose/α–galactose-containing epitope expressed by the medium size subset of sensory afferents.
mAb Laz 141	recognizes CE3, the β–galactose-containing epitope expressed by a small subset of sensory afferents.
Man-BSA	Albumin, Bovine-p-Aminophenyl-α–methyl-mannopyranoside
MW	molecular weight

N-CAM	neuronal cell adhesion molecule
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
TX-100	Triton X-100

INTRODUCTION

In many developing sensory nervous systems, neuronal connections are formed as the initially diffuse axonal innervation of a target region is sculpted or structuralized into columns, layers or other subregions. The sensory information is thus channeled into multiple target regions in the central nervous system. Some of these target regions are exclusively innervated by just one sensory modality, while other target regions are multimodal, receiving more than one type of sensory input. Frequently, neurons conveying these different sensory modalities are identifiable by their different cell surface glycoconjugates. Examples include frog olfactory neurons (Key & Akeson, 1991) and rat and leech mechanosensory and visual neurons (Dodd & Jessell, 1985; Jessell, Hynes, & Dodd, 1990; Peinado, Macagno, & Zipser, 1987; Zipser, Erhardt, Song, Cole, & Zipser, 1994b). The fact that sensory afferents, marked with different cell surface oligosaccharides, ultimately innervate different target regions provides the opportunity to study the role these carbohydrate markers play in formation of neuronal networks.

It has proven difficult to directly study the physiological roles of neuronal carbohydrate markers and the molecular mechanisms of the carbohydrate interactions involved in the formation of neuronal connection, due to the requirement that molecular manipulation experiments must be performed on an intact nervous system. It has

been observed in many experiments that neurons regress when deafferented from their normal connections and subsequently express carbohydrate markers that are characteristic of undifferentiated neuroblasts (Scott, 1993; Dodd & Jessell, 1985; Oudega, Marani, & Thomeer, 1992). Preserving the intact structure of the nervous system is therefore critical for the preservation of normal neuronal carbohydrate profiles. Neither brain slices or primary dissociated neurons or neuronal cell lines, appear to lend themselves to the investigation of the role of surface carbohydrates in the formation of precise connections between neurons. So far, mutant Drosophila, with missing or modified cell surface carbohydrate molecules, have been used to study the importance of carbohydrate markers in the establishment of correct neuronal connections during development of the nervous system (Whitlock, 1993). However, to understand the mechanisms by which carbohydrate markers mediate axonal targeting, it is useful to study their physiological function in a normally developing, intact nervous system.

Like their vertebrate counterparts (Dodd & Jessell, 1985; Riddle, Wong, & Oakley, 1993; Scott, Patel, & Levine, 1990), in the adult leech Hirudo medicinalis, functional sets and subsets of sensory neurons are specifically marked with different glycoconjugates (Table 1) (Peinado, et al., 1987; Peinado, Zipser, & Macagno, 1990; Bajt, Schmitz, Schachner, & Zipser, 1990b; Bajt, Cole, & Zipser, 1990a). The full set of leech sensory afferent neurons can be identified via a mannose-containing epitope, their generic marker CE0 (Carbohydrate Epitope 0) recognized by

Table 1. Carbohydrate markers of leech sensory afferents

Sets or subsets	Epitopes	MAbs	Functions
Full set	CE0	Lan3-2	all sensory modalities
Large subset	CE1	Laz2-369	putative mechanodetectors
Medium subset	CE2	Laz7-79	putative chemodetectors
Small subset	CE3	Lan2-3	putative heat detectors

monoclonal antibody Lan3-2), while disjoint subsets of these neurons correlating with different sensory modalities can be identified via three other carbohydrate epitopes: the subset markers CE1, CE2, and CE3, recognized by monoclonal antibodies Laz2-369, Laz7-79 and Lan2-3, respectively. All of these neuronal markers are located on N-linked carbohydrate chains of 130 kD cell surface proteins (Bajt et al, 1990).

During embryogenesis, the *Hirudo medicinalis* embryo provides a serial array of developmental time points due to the anterior to posterior developmental gradient of its 32 similar segments (Fernandez & Stent, 1982; Weisblat, Harper, Stent, & Sawyer, 1980). Each segment contains one CNS ganglion which is innervated by two bilaterally paired tracts of sensory afferent axons whose cell bodies are associated with epithelial layers. In the course of leech embryonic development, a set of sensilla (a cluster of sensory organs) comes to lie on the middle annulus of each segment on the body wall. These sensilla send their axons to join the different peripheral afferent nerves which are growing toward the CNS in different sequences. Leech sensory afferents that are associated with epithelial layers transduce different sensory modalities (Peinado, et al., 1987; Zipser, Erhardt, Song, Cole, & Zipser, 1994a). Afferents' surface carbohydrate epitopes CE1, CE2, and CE3 delineate disjoint subsets of these sensory neurons: putative mechano-, chemo-, and heat detectors.

The projections of sensory afferents undergo characteristic morphological changes during the formation of neuronal networks. Previously, it had been shown (Zipser, Morell, & Bajt, 1989; Zipser

and Cole, 1990) that during embryogenesis sensory afferents defasciculate and diffusely disperse across the CNS neuropil after having tracked through peripheral nerves as tight axonal bundles. In contrast, in the CNS neuropil of adult leeches, CE1, CE2 and CE3 axons project differentially into several separate target regions in the sensory neuropil (Figure 1) (Peinado, et al., 1987; Peinado, et al., 1990; Zipser et al,1994).

The morphological changes of the sensory afferent projections in the CNS are mediated by a two-step process. In the first step, sensory afferent axons defasciculate and disperse across the neuropil after having tracked as tight bundles through peripheral nerves. In the second step, subsets of sensory afferent axons segregate into specific target regions. The first step, the projection and defasciculation of sensory afferents in the CNS, has been demonstrated in this laboratory to be regulated by a mannose-specific recognition event involving the fullset carbohydrate marker CE0 (Zipser, et al., 1989). The current thesis work demonstrates that the second step, commitment of subsets of sensory afferents to different target regions, is regulated by carbohydrates marking these different subsets. The studies described here were carried out with cultured embryos of the leech *Hirudo medicinalis*, a model system that allows the intact nervous system develop nearly as normally as in vivo.

The dissertation is composed of three chapters. Chapter One describes development of an in vitro model for sensory nervous system growth in the embryonic leech and characterizes the parameters for assessing afferent differentiation and the kinetic

Figure 1. Diagrammatic representation of the leech sensory afferent system. The cell bodies of bipolar sensory afferents occur beneath the epithelia layer of the skin or gut, either singly (A) or clustered into sensilla (B). Within a sensory afferent, an environmental signal is transduced via a specialization (e.g., cilia) of its apical process (x), and this afferent signal projects directly to the synaptic areas of the CNS (C) synaptic neuropil via a axon (arrows). Within the synaptic neuropil (asterisk), afferents bifurcate, projecting anteriorly and posteriorly, and display en passant synapses (Fernandez, 1978). D, Diagrammatic representation of a cross section taken at ganglionic site (n) illustrates the four sensory neuropil tracts (SNT) into which sensory afferents group their processes as they are visualized through staining for fullset carbohydrate epitope CE0 with mAb Lan3-2 (Peinado, Zipser, & Macagno, 1990): a ventral sensory neuropil tract (vSNT), a dorsal sensory neuropil tract (dSNT) and a pair of medial sensory neuropil tract (mSNTs). In the vSNT, CE1 axons predominate; in the dSNT, CE1 and CE2 axons are spatially overlapping; in the mSNTs, CE1 axons occupy the lateral pole, and CE2 and CE3 axons are spatially overlapping in the medial pole.

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features of molecular perturbation of afferent projection. The studies described in Chapters Two and Three analyzed the roles of the carbohydrate markers CE1 and CE2 in the projection of two subset of afferents to their target regions in the intact nervous system. In Chapter Two, the carbohydrate marker CE1 was shown to contain galactose. The temporally sequential expression of carbohydrate markers, fullset CE0 followed by subset CEs, determined the morphological changes of afferent projections in the CNS neuropil from the first step, defasciculation, to the second step, commitment to the target regions. In Chapter Three, we further described the roles of subset carbohydrate markers CE1 and CE2 in determining the CE1 and CE2 subset afferents committing to their specific target regions. The final structuralization of neuronal connections is regulated by interactions involving the different carbohydrate markers that the subset afferents carry.

CHAPTER 1. KINETICS OF THE INHIBITION OF AXONAL DEFASCICULATION MEDIATED BY CARBOHYDRATE MARKERS IN THE EMBRYONIC LEECH.

In this chapter, we describe the development of sensory afferents and the molecular mechanisms directing the early step of targeting of sensory afferents in the embryonic leech. First, we determine the rate of sensory afferent development in vivo, and then we devised a culture system that permits the normal patterning of their axonal projections in the CNS and PNS to proceed at almost the normal rate. Using this in vitro system, we studied the kinetics of carbohydrate-mediated interactions that regulate the targeting of sensory afferents. An early step in their targeting is the defasciculation and dispersion of axons across the synaptic neuropil. This dispersion of single axons is mediated by their mannose-containing surface epitope (Zipser, et al., 1989; Zipser & Cole, 1991). Culturing embryos in Fab fragment directed against this mannose-containing epitope leads to the inhibition of axonal defasciculation. Here we demonstrate that the rate at which defasciculated axons collapse back into larger axon bundles can be modeled by a first order decay reaction. This suggests that the collapse of each axon into a large axon bundle is an independent event, and, furthermore, that it is not likely to be diffusion limited. This suggests that the early defasciculation step in axonal targeting enables each neuron to project autonomously across the neuropil in the search of its appropriate postsynaptic partners.

INTRODUCTION

The molecular mechanisms underlying the formation of neuronal connections in the central nervous system have been the subject of many hypotheses (Sperry, 1963; Zipser & McKay, 1981; Goodman & Shatz, 1993; Easter, Purves, Rakic, & Spitzer, 1985) for which there is as yet little experimental evidence. One approach to obtain experimental evidence has been to map the trajectories of axons in order to identify potential positional clues that they may follow. However, in the retinotectal system, where many of these studies were carried out, the data on positional clues are inconclusive because axons display different behaviors in vivo and in vitro (Nakamura & O'Leary, 1989; Cox, Mueller, & Bonhoeffer, 1990; Simon & O'Leary, 1992).

Another approach to investigate mechanisms of neuronal connectivity has been to isolate molecules that are expressed in intriguing spatial or temporal patterns in the nervous system (Harrelson & Goodman, 1988; Katz, Moats, & Jan, 1988; Levitt, 1984; Key & Akeson, 1991; Trisler, Schneider, & Nirenberg, 1981; Trisler & Collins, 1987; McLoon, 1991; Zipser & McKay, 1981). To date, the strongest candidates for cell type-specific markers are oligosaccharides. For example, sets and subsets of neurons that correlate with particular sensory functions are identifiable by their unique surface glycoconjugates in vertebrates, insects and annelids (Dodd & Jessell, 1985; Whitlock, 1993; Zipser, Erhardt, Song, Cole, & Zipser, 1994; Peinado, et al., 1990; Riddle, et al., 1993; Scott, et al.,

1990). A difficulty in studying the developmental significance of these carbohydrate markers is that they are very sensitive indicators of the physiological state of a cells. Partially dissociated neurons in brain slices or isolated neurons in dissociated culture lose their specific carbohydrate markers and, instead, express anachronistic surface carbohydrates that in some case were shown to resemble those of undifferentiated neuroblasts (Naegele & Katz, 1990; Dodd & Jessell, 1985; Oudega, et al., 1992; Barakat, Bezamahouta, Zanetta, & Vincendon, 1989). Thus, there are a variety of problems with studying molecular mechanisms of axonal targeting in reduced systems.

Here we have combined cellular and molecular approaches of studying neuronal connectivity using the embryonic leech as a physiological system. Culturing the intact germinal plate of the leech embryo in defined growth medium permits sensory afferent neurons to innervate their target regions normally. After having projected through peripheral nerves in tightly bundled axon tracts, sensory afferents defasciculate into the synaptic neuropil of the CNS ganglia. Previously, we showed that this early defasciculation step is mediated by a mannose-containing epitope on their cell surface (Zipser, et al., 1989; Zipser & Cole, 1991). Here we provide evidence that the inhibition of axonal defasciculation by this method can be modeled by a first order decay process. The kinetics of axonal fasciculation suggest that a defasciculated axon has become an autonomous agent and is therefore capable of independently searching for and meeting its appropriate postsynaptic partners.

MATERIALS AND METHODS

Breeding of embryos and preparation of germinal plates

All experiments were performed on individuals of the leech species *Hirudo medicinalis*, which were bred at room temperature (23°C) in dilute artificial sea water (Forty Fathoms 0.5 gm/liter distilled water) (Marine Enterprises, Inc., Baltimore, ML), and fed with fresh bovine blood. After isolation for 2 months at 15°C, adult leeches were brought together in pairs for mating for about 1 month at room temperature. The gravid leeches were then placed in plastic boxes containing moist sphagnum moss. Boxes were checked daily for cocoons. Once found (day 0), the cocoon was transferred to an incubator at 20°C.

Embryos of a desired age (8 to 14 d) were removed from their cocoons. The epithelial envelope of the embryo was then opened by a dorsal incision to remove the yolk, that exposed the germinal plate attached to the ventral part of the envelope. The germinal plates of sibling embryos (12-18) of a given cocoon were divided into different categories. Each category contains at least three siblings. The germinal plates were then stretched out by pinning their epithelial envelope on UV-sterilized Sylgard (184 silicone, Dow Corning Co. Midland, MI; Cat. # ET053572)-coated culture dishes (35 X 10) (Corning Glass Works. Corning, NY).

Improvement of tissue culture condition for growth of sensory afferents

After embryos were pinned out into culture dishes, they were washed three times with sterile culture medium Leibovitz-15 (L-15) (Gibco Lab., Life Tech. Inc. Grand island NY; Cat. # 0312). The germinal plates belonging to the same category were cultured at 20°C in the same dish in 1 ml of the growth medium L-15 containing 1% ITS+ (insulin, trasferin, selenium, bovine serum albumin, linoleic acid) (Collaborative Res. Inc., Bedford, MA; Cat. # 40352), 1% Penicillin-Streptomycin (Gibco Laboratories, Grand Island, New York), 1% L-glutamine (Gibco Laboratories), 100 μM Vitamin C (Sigma, St. Louis, MO), and 0.15% glucose. This growth medium was first introduced by Zipser et al. (1989). To further improve tissue culture condition, growth factors, such as nerve growth factor (NGF) (Sigma; Cat. # N-0513), epidermal growth factor (EGF) (Collaborative Res. Inc., Cat. # 40001), and β–Endorphin (Peninsula Lab. Inc., Cat. #8609), and potassium were added to the culture medium. The effects of supplements, added individually or in combinations, were determined by measuring the rate of sensory afferent differentiation in the cultured germinal plates. The final enriched growth medium that we used for culturing leech germinal plates consists of the original growth medium further modified to contain 10 mM of potassium, 1nM NGF and 10 nM EGF.

Monoclonal antibodies and their Fab fragments

Monoclonal antibodies (mAbs) used for this study were generated in this laboratory (Zipser & McKay, 1981). MAbs Lan3-2 binds to carbohydrate epitopes (CE0) on the surface of fullset sensory afferent neurons in leeches. MAb Laz1-1 binds to an internal antigen of sensory afferents in leeches. For staining, mAbs were used as supernatant (20 µg/ml), diluted at 1/20.

Fab fragments of mAb Lan3-2 were prepared from the mAbs ascites fluid according to the description by Harlow and Lane (1988). It is preferable to use Fab fragments instead of whole IgG antibodies in perturbation experiments, because whole IgGs with two antigen binding sites can cross-link membrane surface molecules and thus might elicit a general distortion of the membrane topology. Cleaving the IgG at the hinge region produces Fab fragments with only one binding site that cannot cross-link molecules. Therefore, a Fab fragment should only inhibit the function of the molecule to which it binds. To generate the Fab fragments, ascites fluid was precipitated with 40% saturated ammonium sulfate with gentle shaking on ice for 24 hr, followed by dialysis against PBS/4 mM EDTA at 4°C for 36 hr. Then the dialyzed ascites was digested with mercuripapain (Worthington Biochem. Co., Freehold, NJ; Cat. # 2489), followed by dialysis again against 5mM Tris-HCl buffer (pH 8.0) overnight at 4°C. The digested ascites fluid was then applied to a 2.5 ml DEAE cellulose (Whatman Biosys. Ltd, Maidstone, England, Cat. # 4057050) column and eluted with a gradient of 0.005 M Tris-HCL, pH 8.0 to 0.3 M NaCl in 0.005 M Tris-HCl, pH 8. . The concentration of Fab

fragments per fraction was estimated assuming that 1mg/ml of immunoglobulin has an absorption of 1.4 at 280 nm, measured by the spectrophotometer (DU series 62, Beckman). As determined on silver-stained sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), Fab fragments of mAbs have a molecular weight of 55 kDa (Harlow & Lane, 1988).

Perturbation experiments

Germinal plates were cultured in the enriched growth medium in the presence of three different concentrations, 20, 50, and 100 nM, of Lan3-2 Fab fragments for different periods of time ranging from 0.5 hr to 24 hr. Each group containing 3 germian plates. Data was presented as Mean $\pm$ SE (n=7). The kinetics of the perturbations were analyzed in germinal plates that were conditioned to culturing with a 6 hr preincubation in the enriched growth medium.

Immunocytochemistry

For staining fixed tissue, primary and secondary antibodies were diluted in 50 mM phosphate-buffered saline (PBS) (pH 8.1) containing 3% bovine serum albumin (BSA) (Sigma; Cat. # A-9647) and 2% Triton X-100 (t-Octylphenoxypolyethoxyethanol, Sigma). Antibody incubations were carried out on a shaker (200 rpm) at room temperature. All rinsing steps except for the last one were carried out with PBS/3% BSA/2% Triton X-100. The last rinse is performed with plain PBS.

For whole-mounted embryos: germinal plates were washed with PBS (pH 7.4), and fixed with 4% paraformaldehyde (Sigma; Cat. # P-0148) for 30 min at room temperature. Embryos were then post-fixed with absolute methanol for 15 min and extracted in absolute xylene for 5 min, followed by rehydration with absolute methanol, 70% ethanol, and PBS. Embryos were incubated overnight with mAb Lan3-2 hybridoma supernatant (1/20). After three rinses, embryos were incubated with biotinylated rabbit-anti mouse F(ab')2 fragments (dilution 1/100) (Dako Co. Carpinteria, CA; Cat. # E413) for 3 hr. After three rinses, embryos were incubated for 4 hr with horseradish peroxidase (HRP) conjugated to avidin (1/150, Vector Lab., Burlingame, CA; Cat. # A-2004), or with fluorescein avidin D (FITC) (1/600) (Vector; Cat. # A2001). After three rinses, the fluorescently stained embryos were mounted in 70% glycerol to which saturating levels of p-phenylenediamine (Sigma; Cat. # P-1519) had been added to prevent fading of fluorescein. Embryos that had been incubated with HRP-conjugated secondary antibodies were preincubated for 10 min in a fresh solution of 0.25 mg/ml DAB (3,3'-diaminobenzidine tetrahydrochloride) (Sigma, Cat. # D-5637). The chromogen reaction was started with a few drops of 0.3% H_2O_2 and terminated by three times washes with PBS. The DAB-stained embryos were dehydrated with 75%, 95%, and 100% ethanol followed by xylene, each step taking 10 minutes. The dehydrated embryos were mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ; Cat. # SP15-500).

Embryonic sections: the dehydrated DAB-stained embryos were treated in Immuno-bed A infiltration solution (Polysciences, Inc.

Warrington, PA) for 3 hr on ice and then were embedded in glycol-methacrylate (Immuno-bed embedding solution, Polysciences, Inc.) in a 1.5 ml micro-centrifuge tube. After 8 hr at room temperature, the blocks were dry, and the specimens were sectioned on a dry glass knife at 2 μm. Sections were then transferred to a glass slide and mounted in Permount.

Data analysis of immunocytochemically stained tissue by confocal microscopy

The fluorescein-stained sensory afferent axons were analyzed under the Odyssey, a Real Time Laser Scanning confocal microscope (Noran instrument, Inc; Madison, WI). Serial options were captured with 25 (Zeiss), 40, 63 or 100 (Nikon) x oil immersion objectives. The laser excitation and primary barrier filters for fluorescein were 488 and 515 nM, respectively. The entire projections of sensory afferents/hemineuropil were captured in 15 serial optical sections taken at 1 μm steps. To reconstruct three-dimensional images or to analyze the data, the optical sections were processed with Image-1 program (Universal Image Co., West Chester, PA).

The DAB-stained tissue was analyzed under brightfield optics using 25, 40, 63, and 100 x Zeiss oil immersion lenses on a Nikon Optiphot. The specimens were also optically sectioned (in 1 μm steps, the laser excitation sources is 488) by using Zeiss 10 laser scanning confocal microscope (Carl Zeiss, Inc.) in the transmission operating mode to produce images that are sharper than those obtained with a research microscope using ordinary light. These

brightfield images captured as a PIC file by the transmission Zeiss laser confocal microscope were then translated through Adobe Photoshop software (Adobe Systems, Inc.) to the TIFF file recognized by Image-1 program. Using Image-1 program, three dimensional images were reconstructed from the serial optical sections for analysis of axon differentiation and perturbation.

Parameters for assaying differentiation and perturbation of sensory afferents

For all of these measurements, sensory afferents were stained with DAB. Each segmental ganglion of the CNS furnished two measurements of sensory afferent differentiation or perturbation because each hemineuropil receives its own sensory afferent projections from the periphery. The stages of sensory afferent differentiation were determined using the innervation of the synaptic neuropil of segmental hemiganglia by sensory afferents arriving from the median, dorsal and connective nerves as parameters.

The perturbation of sensory afferent projection in response to the application of Fab fragments was measured bilaterally in the 10 most posteriorly innervated segmental ganglia of the leech CNS in the developing germinal plate. Single axons and axonal tracts in the neuropil were hand-counted under the brightfield microscope (63 x Zeiss oil immersion objective). In embryos (10 to 11 d) in vivo, and cultured in enriched medium, sensory afferents defasciculate into single axons that disperse across the neuropil in these ganglia (the

more anterior ganglia of the CNS are not included in the analysis because in those ganglia subsets of sensory afferents have already begun to group into discrete target regions; Song and Zipser, submitted). The exposure to Fab fragments typically elicits the collapse of defasciculated sensory afferents into 1, 2 or 3 thick axonal tracts (Zipser et al, 1989; Zipser and Cole, 1990). This pattern is also seen with a low frequency in vivo or in germinal plates that are cultured in enriched medium without Fab fragments (6.5% of sensory afferent projections in 600 hemineuropils of the ten most posteriorly innervated ganglia). We therefore assumed that for any observed perturbation in the presence of Fab fragments, 6.5% were due to other causes and we subtracted 6.5% from all of the perturbation measurements.

The kinetics of the inhibition of sensory afferent defasciculation elicited by Fab fragments were studied in sibling germinal plates (11d) that were cultured, following a 6 hr preincubation, for 1 to 6 hr in either enriched growth medium alone (no-treatment) or in enriched growth medium to which 50 nM mAb Lan3-2 Fab fragments had been added. Sensory afferent projections were optically sectioned at 1 μm step (10 sections, Zeiss confocal microscope) in the bilateral neuropils of midbody ganglia 11, which in these experiments were the 10th most posteriorly innervated ganglia of the CNS. The full sensory afferent projection of each hemiganglion was reconstructed by overlaying the 10 serial optical sections using Image-1. The perturbation of sensory afferents in a given hemiganglion was measured by hand-counting the number of separate projections consisting of either single axons or axonal

tracts in the 20 x 40 μm (lateral to medial x anterior to posterior) area of a given hemineuropil.

RESULTS

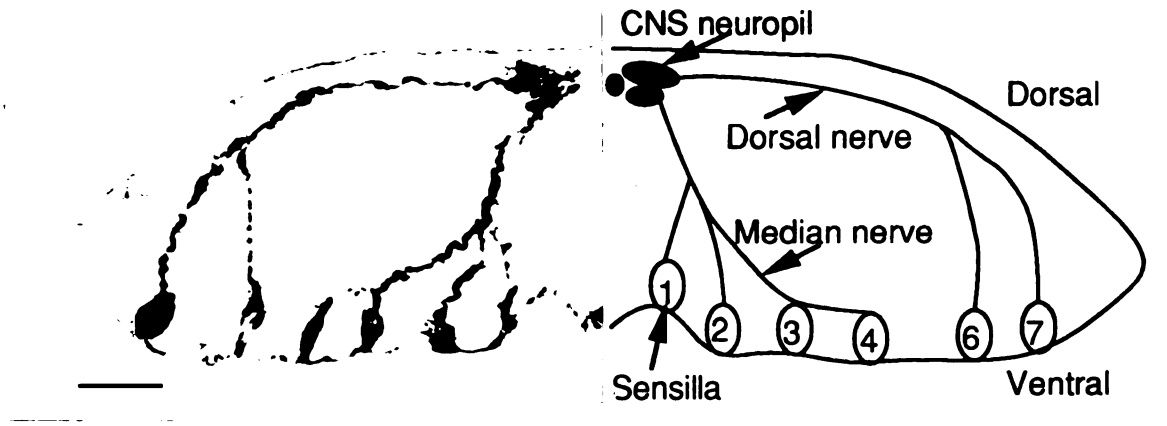
Normal development of leech sensory afferents

Configuration of the sensory afferent system during early embryogenesis

Leech sensory afferents appear after one fourth of embryogenesis has been completed. Their development can be followed via two different cell type-specific markers, both of which are expressed constitutively. Sensory afferents express on their surface a mannose-containing epitope (CE0, carbohydrate epitope 0) that is reactive with a panel of different monoclonal antibodies, among them mAb Lan3-2 (Zipser & McKay, 1981; Peinado, et al., 1987). Furthermore, an early differentiating subset of these sensory neurons in addition expresses an internal antigen recognized by mAb Laz1-1 (Stewart, Gao, Peinado, Zipser, & Macagno, 1987; Moore, Morell, & Zipser, 1988).

As illustrated in a cross section (Figure 2) the germinal plate of an 11 day old embryo contains six sensory organs, or sensilla, in which the cell bodies of sensory afferents are found. The sensilla are located below the ventral skin extending from the midline to the lateral edge of the germinal plate. Axon bundles emanating from these ventral sensilla project dorsally into the synaptic neuropil of the CNS ganglion via two peripheral nerves, the median and dorsal nerves. Axon bundles from sensilla 1 - 4 ascend near the midline via

Figure 2. The cell bodies and axons of sensory afferents in a cross section of the leech germinal plate. The axonal pathways of sensory afferent projection, stained with mAb Lan3-2, are illustrated in the germinal plate of an 11 day embryo, cross sectioned at midbody ganglion 10. The image is captured by the Zeiss confocal microscope and reconstructed with Image-1 software. The cell bodies of sensory afferents are clustered in sensory organs, the sensilla (15 μm in diameter), below the ventral surface of the germinal plate. From the midline toward the lateral edge of the germinal plate, sensilla 1, 2, 3, 4, 6, and 7 are spaced at about 40 μm intervals. Afferents from sensilla 1 to 4 project their axons via the median nerve into the dorsally located segmental ganglion; afferents from sensilla 6 and 7 project their axons through the dorsal nerve. The peripheral path through the dorsal nerve (average of 240 μm) is longer than the path through the median nerve (average of 110 μm). Bar, 50 μm.


CNS neuropil
Dorsal
Dorsal nerve
Median nerve
1
2
3
4
6
7
Sensilla
Ventral

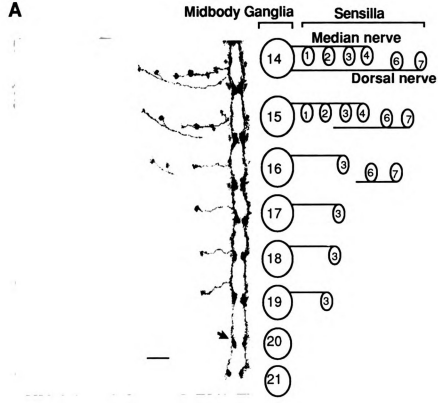
the median nerve while axon bundles from sensilla 6 - 7 ascend near the lateral edge and then project medially via the dorsal nerve.

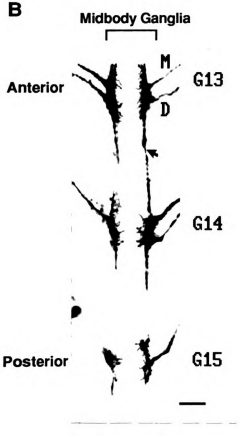
Spatial gradient of sensory afferent differentiation

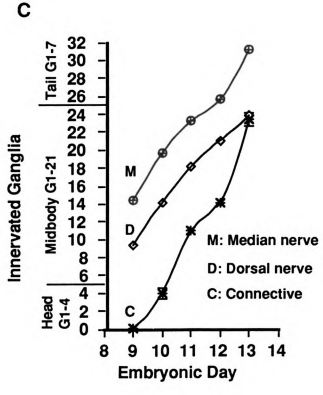
The spatial sequence in which different components of the sensory afferent system develop extends across several embryonic body segments as illustrated in the wholemount view of an 11 day old germinal plate (Figure 3A). Both the sensory afferents and a transient cell (arrow) that serves as a convenient marker for segmental ganglia were stained with mAb Laz1-1. Sensory afferents develop along an anterior to posterior gradient. The first sensory afferents are born in sensillum 3, as seen in the posterior ganglia 19 to 17. Usually, as soon as sensillum 3 has appeared, sensory afferent axons are detected in its segmental ganglion (86 % of 50 embryos). The difficulty in monitoring the growth of a sensory afferent axon bundle through the median nerve is presumably due to the short distance (average 110 μm; n = 50) between the newly differentiated sensillum 3 and its segmental ganglion. Thus, the projection of median nerve afferents into the CNS serves as an indicator for the first sensory afferent differentiation in a segment. After sensillum 3 has appeared, the other sensilla differentiate in a defined spatial sequence as illustrated in Figure 3A. Later during embryogenesis, sensillum 4 will divide giving rise to sensillum 5. Axon bundles emanating from sensilla 6 and 7 can be seen growing towards the CNS in two consecutive segments. The pathway from sensilla 6 and 7 to their segmental ganglion through the dorsal nerve is about 220-

Figure 3. Normal differentiation of sensory afferents.
A. The spatial gradient of afferent differentiation: The anterior-posterior gradient of sensory afferent development is illustrated in a wholemount view of midbody ganglia 14 to 21 in the germinal plate of an 11 day old embryo stained with mAb Laz1-1. Besides staining sensory afferents, the mAb Laz1-1 also stains a transient cell (arrow), which serves as a convenient marker for the embryonic ganglia (Stewart, Gao, Peinado, Zipser, & Macagno, 1987). Because posterior segments develop later than the anterior segments, they demonstrate the site where sensory afferents are first born, which is in sensillum 3 (projecting here to midbody ganglia 19, 18, 17). In most cases, as soon as sensory afferents are detected in sensillum 3, their axons are also detected in the respective segmental ganglion, presumably because the path from sensillum 3 to the CNS is only 110 μm long at this stage. Sensilla 6 and 7 differentiate later which translates into a spatial delay of 4 segments (midbody ganglion 16). However, their axons do not enter the CNS until two segments later (midbody ganglion 14) because the dorsal nerve pathway is significantly longer than the median nerve pathway. The last sensilla to differentiate are sensilla 1, 2 and 4. Spatially, the differentiation of the six sensilla extends over six midbody segments. Bar, 50 μm.
B. The formation of the intersegmental connective: The intersegmental projections of sensory afferents are illustrated in a wholemount view of the midbody segments of an 11 day old germinal plate. The connective pathway is visualized by staining embryos with mAb Lan3-2. The main intersegmental pathway of sensory afferents runs through the connective which links the synaptic neuropils of neighboring segmental ganglia. Connectives are formed only after the neuropil is innervated by both the median (M) and dorsal nerve (D), as shown here at the midbody ganglia 14 level. Afferents do not project into the connective from the midbody ganglia 15 where the neuropil is only innervated by median nerve afferents. Bar, 20 μm.
C. The temporal gradient of afferent differentiation: We determined the temporal gradient of sensory afferent differentiation in 8 to 13 day embryos (n=120) stained with mAb Lan3-2. We used three separate measurements to determine the state of sensory afferent differentation in a given segment: the median nerve projection into CNS neuropil, the dorsal nerve projection into CNS neuropil, and the formation of interganglionic connection. Sensory afferents began differentiating in 8 day old embryos. By embryonic day 9, the median

nerve afferents had innervated half of the segmental chain of 21 midbody ganglia, and by embryonic day 12, they had innervated all 21 midbody ganglia. The innervation of midbody ganglia by the dorsal nerve afferents lags behind 4 to 6 ganglia which is 1 to 1.5 embryonic days of development. The connectives are formed one to two embryonic days after the axons from the dorsal nerve enter the CNS neuropil. The first intersegmental connective is formed between 9 to 10 embryonic days. As the embryo continues developing, connectives are formed at a speed of 5 to 6 ganglia per day. The data in the graph represent Mean $\pm$ SE, analyzed on the software Microsoft excel. The error bars are hardly seen on the graph because of the narrow distribution of the data and the big number of specimens.


A
Midbody Ganglia
Sensilla
Median nerve
Dorsal nerve
14
15
16
17
18
19
20
21
1
2
3
4
6
7


B
Midbody Ganglia
Anterior
Posterior
M
D
G13
G14
G15


C
Innervated Ganglia
Tail G1-7
Midbody G1-21
Head G1-4
M
D
C
M: Median nerve
D: Dorsal nerve
C: Connective
Embryonic Day
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
8
9
10
11
12
13
14

270 μm long at that stage. Dorsal nerve afferents reach their segmental ganglia six segments more anteriorly than median nerve afferents. Thus, the innervation of dorsal nerve afferents serves as an indicator that the major peripheral pathways have been established. Together, the major peripheral components of the sensory afferent system, namely the first six sensilla and the median and dorsal nerves, have differentiated across a sequence of six body segments.

The formation of the central pathway through which connective afferents innervate neighboring segmental ganglia is studied by staining sensory afferents for CE0, their mannose-containing epitope (Figure 3B). Three successive midbody ganglia are used to illustrate the stage at which connective afferents complete their intersegmental pathway. Afferents do not project into the connective at the earliest stage, illustrated in segmental ganglion G15, where the neuropil is solely innervated by median nerve afferents. Later, illustrated in G14 and G13 where the right neuropils are innervated by both median and dorsal nerve, afferents project into the right connectives. This leads to the completion of the intersegmental pathway through the connective between these two ganglia (arrow). As determined by confocal microscopic analysis of serially sectioned neuropil, sensory afferents begin to assemble in their stereotypic target regions in the embryonic ganglia that are joined via connective afferents (Song & Zipser, submitted, a; Song & Zipser, submitted, b; Zipser, et al, 1994). Thus, the completion of the intersegmental pathway via connective afferents serves as an indication that sensory afferents have begun their synaptogenesis.

This sequence of three ganglia also demonstrates that sensory afferents in the right and left body segments differentiate independently of each other.

Temporal gradient of sensory afferent differentiation

To determine the relationship between the spatial gradient of sensory afferent differentiation and the absolute timing of this differentiation, we used each body segment to obtain two independent measurement of our three parameters of sensory afferent differentiation: the ganglionic innervation by median, dorsal and connective afferents.

The time course of sensory afferent differentiation was studied in 8 to 13 day old embryos (n = 120). Both the head and the tail regions are innervated at a more rapid intersegmental rate by median and dorsal nerve and connective afferents than the midbody ganglia (Figure 3C). There is no significant difference for the rate of innervation by the median and dorsal nerve afferents of the midbody ganglia (4 segmental ganglia/day) , although in each case, a given segmental ganglion is innervated by the median nerve 1 to 1.5 days earlier than by the dorsal nerve. The first intersegmental connections are formed by sensory afferents between embryonic day 9 and 10. This ganglionic innervation by connective afferents proceeds rapidly and catches up with dorsal nerve innervation in posterior midbody ganglia.

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Devising a culturing method for the leech germinal plate

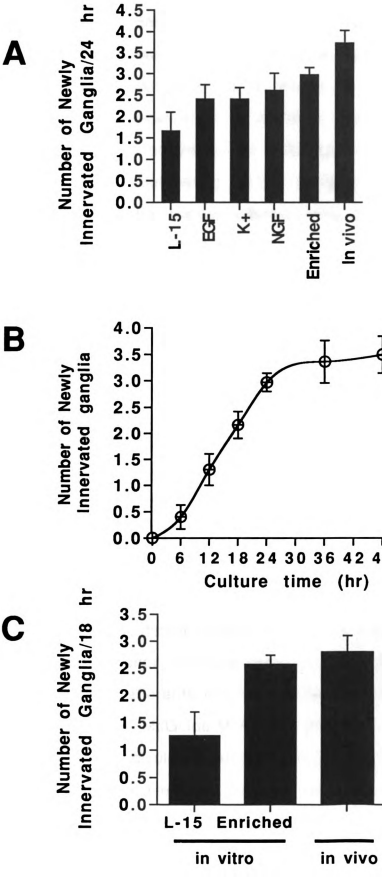
Importance of growth factors

We improved our culture system for the leech germinal plate using the normal developmental clock just described based on the median nerve innervation as a clock against which to measure development in culture. We assayed various culture modifications to determine which produced a developmental sequence that was more faithful to the original. Culturing germinal plates of 10 to 11 day embryos in our original growth medium (Zipser, et al., 1989; Zipser & Cole, 1991) decreases the rate of ganglionic innervation to 44.5% of the normal rate in vivo, that is only 1.7 instead of 3.7 segmental ganglia were innervated per 24 hr. Adding epidermal growth factor (EGF) (10 nM), increasing potassium to 10 mM, or adding nerve growth factor (NGF) (1nM), improved that rate of ganglionic innervation to 64.3 to 69.7% of the normal rate. Combining the three separate treatments further improved the rate of ganglionic innervation to 79.6% of the normal rate (Figure 4A). Another reagent, β–endorphin, similarly improved the rate of ganglionic innervation but it did not add to the effect produced by supplementing the growth medium with NGF, EGF and elevated potassium combined.

Importance of time of culturing

A further improvement of our in vitro system came from the finding that the rate of ganglionic innervation by sensory afferents

Figure 4. Differentiation of sensory afferents in the tissue culture system as assessed by development of the median nerve.
A. Effects of growth factors: The addition of growth factors has been used to try to improve the tissue culture condition of the leech embryos. Culturing 10 to 11 day embryos in L15 only supplemented with insulin, transferrin and selenium reduced the differentiation rate of the median nerve afferents by 55.5%. The rate of segmentally ganglionic innervation dropped from normally 3.73 ± 0.3 ganglia/24 hr (n=120) to 1.66 ± 0.44 ganglia/24 hr (n=30). Adding 10 nM of EGF (n=10) or increasing potassium to 10 mM (n=10), increased the segmental innervation to 2.4 ± 0.3 ganglia/24 h; adding 1 nM of NGF increased the rate of innervation to 2.6 ± 0.41 ganglia/24 hr (n=10); NGF, EGF and increased K^+ in combination increased the segmental rate of innervation to 2.97 ± 0.17 ganglia/24 hr (n=30), which is 79.6 % of the normal differentiation rate.
B. Optimal time window for growth in culture: Embryos were cultured in the enriched medium for 48 hr and sensory afferent development was measured by determining the rate with which median nerve afferents innervated segmental ganglia at 6 hr intervals (each time point data was collected from 10 to 30 embryos). During the first 6 hr in culture, there was very little sensory afferent development (0.4 ± 0.23 ganglia/6h). Median nerve afferents innervated less than half of a segmental ganglion. During the following 18 hr, a steep increase took place in sensory afferent development. Median nerve afferents innervated segmental ganglia at the average rate of 0.86 ganglia/6 hr which is 92% of the normal rate of 0.93 ganglia/6 hr. But after 24 hr of culturing, sensory afferent development virtually ceased (rate of 0.13 ganglia/6 h).
C. The final tuning of culture conditions: The final improvement in our culture system came from the observation that the fastest differentiation rate occurred during the culturing period from 6 to 24 hr as shown on Figure 3B. Comparing the differentiation rate during this 18 hr culturing period (2.57 ± 0.17 ganglia) with the normal rate (2.8 ± 0.3 ganglia) shows that the in vitro rate of differentiation is 92% of that seen in vivo, which is also 51.8% more than when cultured in medium L-15 (1.26 ± 0.44 ganglia) without the addition of growth factors.


A
Number of Newly Innervated Ganglia/24 hr
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
L-15
EGF
K+
NGF
Enriched
In vivo
B
Number of Newly Innervated ganglia
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 6 12 18 24 30 36 42 48
Culture time (hr)
C
Number of Newly Innervated Ganglia/18 hr
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
L-15 Enriched
in vitro
in vivo

is a function of time in culture (Figure 4B). During the first 6 hr of culture, there is very little new innervation of segmental ganglia. However, during the following 18 hr of culture, sensory afferents newly innervate segmental ganglia at 91.8% of the normal rate. Afterwards, ganglionic innervation by sensory afferents suddenly declines. The improved conditions for culturing the leech germinal plate are illustrated by comparing normal ganglionic innervation (in vivo) with that of germinal plates cultured using our original (L15; - preincubation) and our new methods (enriched; + preincubation) (Figure 4C). Thus, using our new methods, the development of sensory neurons in culture, as measured by the ganglionic innervation of median nerve afferents over an 18h time window, has doubled and nearly reached a normal rate.

Kinetics of the inhibition of sensory afferent defasciculation

Dose and time dependency of the inhibition of axonal fasciculation by Fab fragments

We used our improved culture system to study the kinetics of the inhibition of axonal defasciculation by Fab fragments directed to CE0, the sensory afferents' mannose-containing epitope. CE0 is expressed by sensory afferents on their entire cell surface (Hogg, Flaster, & Zipser, 1983; McGlade-McCulloh, Muller, & Zipser, 1990). Axons that track as fasciculated bundles through median and dorsal nerves and the connective express CE0 on their cell surface. Likewise, axons that defasciculate into the synaptic neuropil express

CE0. Thus, CE0 could mediate the fasciculation or bundling of axons during their pathfinding through nerves, but it could also function to mediate the defasciculation or unbundling of axons during their targeting in the synaptic neuropil. Three separate lines of experimental evidence indicate that CE0 mediates axonal defasciculation rather than axonal fasciculation (Zipser, et al., 1989; Zipser & Cole, 1991). This implicates the CE0 epitope in axonal targeting in the synaptic neuropil rather than in axonal pathfinding along nerve tracts.

To determine appropriate concentrations of Fab fragments and exposure times to study the kinetics of the inhibition of axonal defasciculation, we first did a dose response curve. The effects of three different concentrations of CE0 Fab fragments on sensory afferent growth were measured at six different time intervals. Germinal plates of sibling embryos were cultured in the enriched growth medium in the absence of Fab fragments or in the presence of 20, 50 or 100 nM of CE0 Fab fragments. Fab fragments were applied at the beginning of culturing. Using seven embryos for each time point and dosage, the projections of sensory afferents were bilaterally examined in the ten most posteriorly innervated segmental ganglia. As shown in Figure 5A, normally developing sensory afferents defasciculate and project their axons diffusely in a semicircular pattern across the ipsilateral neuropil (I). In contrast, sensory afferents that are perturbed by Fab fragments grow in one, two or three thick axonal tracts from which an occasional single axon or thin axonic fascicle may issue (II) (Zipser & Cole, 1991). The presence of Fab fragments in the neuropil both inhibits

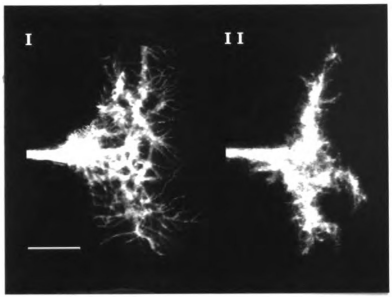
Figure 5. Perturbation of afferent projections by CE0 mAb Lan3-2 Fab fragments.

A. Afferent projection pattern in CNS neuropil and its perturbation: Normally, upon entering the CNS from peripheral nerves, the bundled sensory afferents defasciculate and amorphously spread throughout a 20 μm diameter of ipsilateral neuropil. Sensory afferents continue to grow as defasciculated, single axons when cultured for 12 hr in enriched growth medium (I). In contrast, culturing embryos in the presence of 40 nM of mAb Lan3-2 Fab fragments inhibits axonal defasciculation. Axons fasciculated into three thick axon bundles (II). Bar, 10 μm.

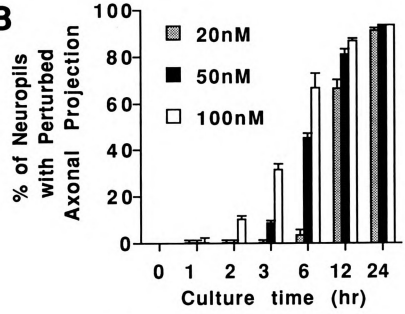
B. Perturbation effects of mAb Lan3-2 Fab fragments: Eleven day old embryos were treated with 20, 50 or 100 nM of mAb Lan3-2 Fab fragments for 1, 2, 3, 6, 12, and 24 hr. The defasciculation of sensory afferents was measured in the absence and presence of Fab fragments, using seven embryos for each time point. The perturbation effect was measured by determining the inhibition of defasciculation bilaterally in the 10 most posteriorly innervated ganglia. Sensory afferent projections were considered to be perturbed if axons fasciculated into one, two or three thick bundles. We already detected perturbation effects within the first 6 hr of culture even though the sensory afferents grow very little during this period. The perturbation exhibts dose-response and time-response effects. During the first 2 hr of culturing, only high concentrations of Fab fragments (100 nM) elicited a noticeable perturbation of 10.2 ±1.5%. An intermediate concentration of Fab fragments (50 nM) elicited 45.1 ± 2% perturbation after 6 hr of culturing. Low concentrations of Fab fragments (20 nM) only elicited perturbations after 12 hr of culturing. After 24 hr of culturing, all concentrations elicited maximal perturbation (>90%).

C. Differentiation rate-dependence of afferents' sensitivity to Fab perturbation: After a 6 hr preincubation period, sensory afferents demonstrated increased sensitivity to 50 nM of Lan3-2 Fab fragments. Inhibition of defasciculation was detectable after a 1 hr treatment with Fab fragments (6.2 ± 1.5%). The percentage of sensory afferent perturbation increased gradually with extended treatment; 80 ±1% perturbation was elicited after a 6 hr exposure to Fab fragments, which is a 44% increase in efficiency compared to the 45.1% perturbation elicited after a 6 hr exposure to Fab fragments without preincubation.

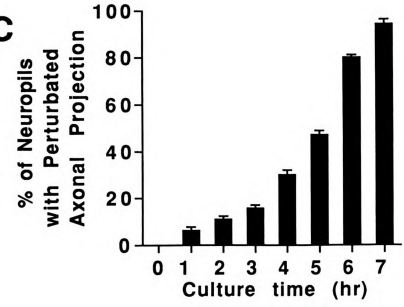
A


I
II

B


% of Neuropils with Perturbed Axonal Projection
100
80
60
40
20
0
20nM
50nM
100nM
0 1 2 3 6 12 24
Culture time (hr)

C


% of Neuropils with Perturbated Axonal Projection
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Culture time (hr)

defasciculation of newly arriving axons and also promotes fasciculation of those axons that had defasciculated prior to the application of Fab fragments (Zipser & Cole, 1991).

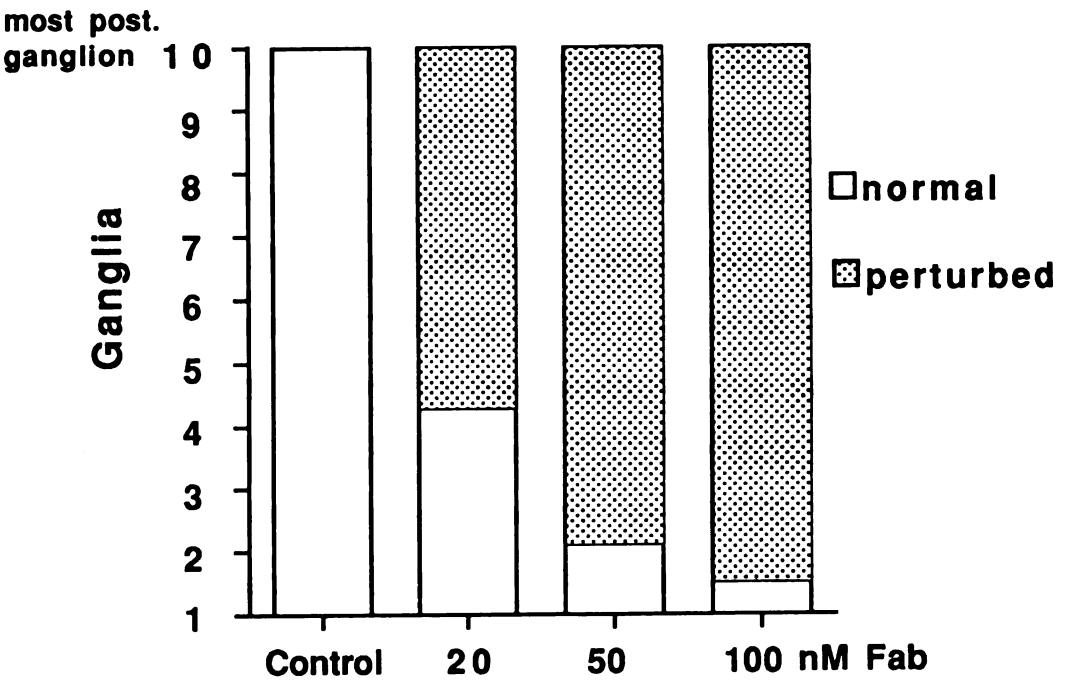
As shown in Figure 5B, all three concentrations of Fab fragments perturbed sensory afferent growth in more than 90% of the hemiganglia after 24 hr of treatment. About 80% perturbation was elicited by 20 and 100 nM Fab after 12 and 6 hr, respectively. The first perturbations were detected after germinal plates were treated for 2 hr with 100 nM or for 3 hr with 50 nM Fab.

To increase the sensitivity of our assay, we repeated the perturbation study during the time window in which sensory afferents are developing at a nearly normal rate (see Figure 4B). Germinal plates were preincubated for 6 hr and then treated with 50 nM of CE0 Fab fragments. After one-hour of Fab fragment treatment, 6% of the sensory afferent projections were perturbed. The percentage of perturbed sensory afferent projections gradually increased with ongoing exposure to Fab fragments reaching nearly 100% after 7 hr. Comparing the effects of a six-hour treatment with 50 nM Fab on sensory afferents that were preconditioned to culturing (80%, Figure 5C) to those that were not (45%; Figure 5B) demonstrates nearly a doubling of the efficiency of Fab perturbation. This comparison suggests that sensory afferents in the synaptic neuropil are more sensitive to the presence of Fab fragments when they are developing at their normal rate.

The age dependency of the sensitivity of sensory afferents to Fab fragments

We next asked whether the sensitivity of the defasciculated axons to Fab fragments was dependent on their developmental age. To answer this question we compared the percentage of axonal perturbations in neuropils that had been innervated during the first 12 hr of culturing in Fab fragments to those that had been innervated prior to culturing. We assumed that 1.3 ganglia had been innervated during culturing based on our data on the rate of innervation of segmental ganglia during the first 12 hr of culture. We found, as shown in Figure 6, that all of the projections into the neuropil that presumably occured during culturing in the presence of Fab fragments were perturbed. In contrast, sensory afferents that projected into the neuropil prior to the application of Fab fragments were differentially affected. At low doses of Fab fragments (20nM), 56% of the previously developed projections were perturbed after 12 hr. At higher concentrations of 50 nM or 100 nM Fab fragments, 77% or 85% of existing projections were perturbed. The results suggested that newly formed sensory afferent projections are more susceptible to the perturbation by Fab fragments. In these experiments, we found that the application of Fab fragments did not reduce the segmental rate of ganglionic innervation by sensory afferents. To the contrary, low concentration of Fab fragments (20 nM) increased the rate of ganglionic innervation by sensory afferents by 33.7%. This increase in the rate of innervation presumably reflects an accelerated

Figure 6. The relationship between axonal perturbation and their state of differentiation. The perturbation of sensory afferent projections in response to 20, 50 and 100 nM Fab fragments were measured in the ten most posteriorly innervated ganglia of the CNS in a given germinal plate. All projections of sensory afferents were perturbed in the most posterior 1. 3 ganglia that were innervated during the 12 hr culturing. However, afferents that had already innervated the rest 8.7 ganglia before the application of Fab fragments are differentially affected. In more than 90% of the cases, the perturbation of axonal projections occurred preferentially in the more recently innervated ganglia. Cultured in 20 nM Fab fragments, 56.4% of the projections (in the 4.9 most posteriorly innervated ganglia) were perturbed; cultured in 50 nM Fab, 77% of the projections (in the 6.7 most posteriorly innervated ganglia) were perturbed; cultured in 100 nM Fab, 84.7% (the 7.4 most posterior ganglia) were perturbed.


most post.
ganglion
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Ganglia
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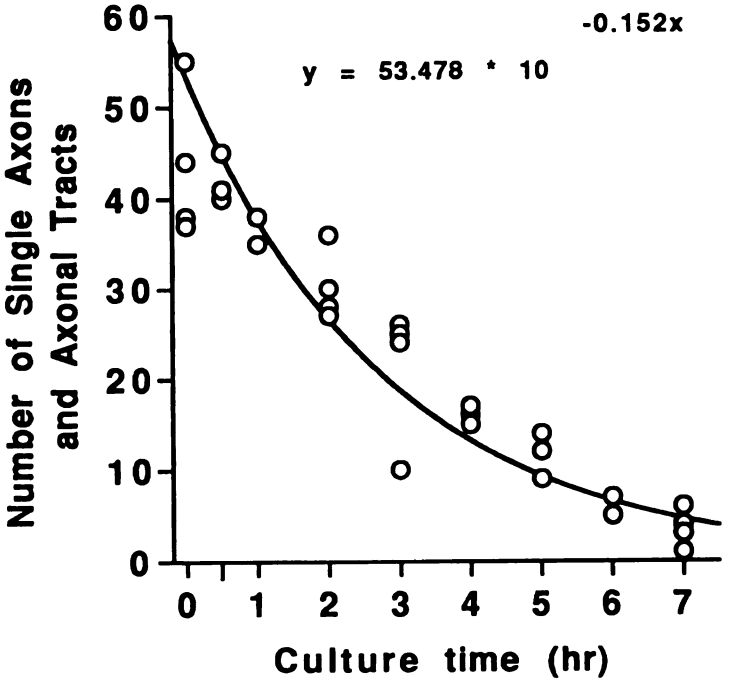
20
50
100 nM Fab

differentiation of sensillum 3 rather than an increase in the rate of axonal growth.

Kinetics of axonal perturbations by Fab fragments

The kinetics of the inhibition of axonal defasciculation were examined by determining the rate at which the defasciculated axons disappeared after the application of Fab fragments. These measurements were performed on the 11 day old germinal plates that had been cultured in the absence or presence of 50 nM Fab fragments following a 6 hr preincubation. In all germinal plates, the number of axons and axonal tracts were counted in the reconstructed confocal images from midbody ganglia 11 in which axons had defasciculated 1.5 day prior to culturing. The number of axons and axonal tracts gradually decreased as the exposure time to Fab fragments increased from 1 to 7 hr (Figure 7). The data points were curve fitted with a first order decay process $y = 53.478 * 10^{-0.152x}$ in which the average number of axons and small axonal tracts (y) in a specific time (x) is a simple function of total axon population. The half life for the maintenance of axonal defasciculation or for the collapse of axons into refasciculated bundles is 1.98 hr. Thus, nearly every 2 hr, 50% of the population of single axons will disappear, by refasciculating which gives rise to either one, two or three thick axonal tracts.

Figure 7. The kinetics of perturbing axons with Fab fragments. The number of single axons and axonal tracts were counted in midbody ganglia 11 of germinal plates that had been cultured in the absence or presence of 50 nM Fab fragments following a 6 hr preincubation. The number of single axons and axonal tracts gradually decreased as the exposure time to Fab fragments increased from 1 to 7 hr. The number of single axons and axonal tracts begins to decrease 0.5 hr after adding mAb Fab fragments. The average number of axons (y) in a specific time (x) is a simple function of total axon population then, as depicted in the equation: $y = 53.478 * 10^{-0.152X}$. The rate (y') at which axons disappear at a certain perturbation culturing time period (x) is exponentially proportional to the number of axons then, as shown by the equation: $y' = -18.73 * 10^{-0.152X}$. The half life for the maintenance of defasciculated axon survival is 1.98 hr.


y = 53.478 * 10 -0.152x
Number of Single Axons and Axonal Tracts
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7
Culture time (hr)

DISCUSSION

We developed an experimental system that allows the investigation of sensory afferent projections in the central nervous system of the leech. We used this system to study the kinetics of molecular mechanisms that mediate axonal targeting in the synaptic neuropil. To develop this experimental system, we first determined the rate of normal sensory afferent differentiation in vivo.

Devising a culture system with nearly normal sensory afferent development

Previously, sensory afferent development had been studied with two monoclonal antibodies that recognize constitutively expressed antigens: mAb Lan3-2 recognizes CE0, a mannose-containing epitope on the surface of sensory neurons (Zipser & Cole, 1991; McGlade-McCulloh, et al., 1990; McKay, Hockfield, Johansen, Thompson, & Frederiksen, 1983; Johansen, Kopp, Jellies, & Johansen, 1992); mAb Laz1-1 which like Lan3-6 and Laz2-1 recognizes an internal antigen (Stewart, Macagno, & Zipser, 1985; Moore et al,). Here we used these two different antigens as independent markers to analyze the normal rate of sensory afferent development in the 32 ganglia of the nerve cord that develop in the germinal plate from five bilateral pairs of teloblasts (M-Q) (Weisblat et al., 1980). We defined three stages of sensory afferent development using as parameters the innervation of segmental ganglia by sensory afferents arriving from peripheral nerves or the central connective. The projection of median nerve

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differ

dorsa

differ

proje

sens

perip

inner

rapid

in ra

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afferents provides an estimate for the onset of sensory afferent differentiation in the respective segment while the projection of dorsal nerve afferents provides an estimate for the completion of differentiation of the six major segmental sensilla. Lastly, projections of connective afferents serves as an indicator that sensory afferents have begun to group into discrete target regions.

The rate at which ganglia are innervated by the afferents from peripheral and central pathways is dependent on the segment. The innervation of the head and tail ganglia proceeds significantly more rapidly than the innervation of the midbody ganglia. This difference in rate may be related to the difference in length of the head and tail segments and the midbody segments. Both head and tail segments are much shorter than the midbody segments. The higher innervation rate in the shorter segments could be explained by the release of growth factors by the differentiating sensilla which diffuse to neighboring segments, where they stimulate the differentiation of the adjacent posterior sensilla.

We used the slower rate of innervation of the segmental midbody ganglia (4 segmental ganglia/ day) as benchmark for developing a culture system for optimal sensory afferent development. In our experiments, we measured the kinetics of sensory afferent projection in these more slowly innervated midbody ganglia.

Our previous method for culturing germinal plates, as we mentioned here, had resulted in a 55% decrease in the rate of sensory afferent development. Enriching the growth medium by adding EGF and NGF as well as increasing the potassium concentration improves

the rate of sensory afferent development. We should note that so far, there is no information on leeches having endogeneous NGF and EGF-like growth factors. The final improvement in culturing conditions came from the realization that the rate of sensory afferent development in culture is a function of time in culture. During the first 6 hr of culturing, sensory afferent development virtually ceases, presumably due to the shock of opening the epithelial envelope, extruding the yolk and the exposure of the intact germinal plate to the defined enriched growth medium. However, after germinal plates are allowed to condition to culture for 6 hr, the development of sensory afferents continues at a 92% normal rate for the next 18 hr. Thus, using an enriched growth medium and making use of an optimal time window for culturing permits nearly normal sensory afferent development in vitro.

Kinetics of sensory afferent projections in the synaptic neuropil

Sensory afferents track through peripheral nerves in tight axonal bundles but then defasciculate as they enter the synaptic neuropil. As a result of this defasciculation, single axons disperse across the neuropil. Our kinetic studies indicate that the inhibition of axonal defasciculation follows a first order decay process. The significance of a first order decay process is that the loss of each single axon occurs independently of all other single axons at any moment. This suggests that each defasciculated axon projects autonomously across the neuropil. The importance of such

autonomous projection is that it allows each axon to independently search for and meet its appropriate postsynaptic partners.

The molecular mechanism of axonal defasciculation consists of a mannose-specific recognition involving the sensory afferents' own mannose-containing surface epitope, CE0, mentioned previously as the marker to define the rate of sensory afferent development. The evidence for a mannose-specific recognition mediating axonal defasciculation is based on three independent lines of experimental evidence (Zipser & Cole, 1991; Zipser, Morell, & Bejt, 1989). The defasciculation is inhibited by (1) Lan3-2 Fab fragments that bind to CE0, the mannose-containing epitope; (2) N-Glycanase that cleaves the N-linked carbohydrate chain on which the mannose-containing epitope is located and (3) mannose-conjugated BSA that competes with the mannose-containing epitope for a hypothetical mannose-binding protein. Any of these perturbations lead to the loss of single, defasciculated axons. Afferents that newly projected into the neuropil did not defasciculate, but rather continued to extend into the connective as newly fasciculated bundles. Defasciculations that had been achieved prior to the experiments disappeared and were replaced by one, two or three axonal tracts, suggesting that the previously defasciculated afferents collapsed back into common axon bundles. Even CE0 is expressed everywhere on the surface of sensory afferents, the most dramatic effect Fab fragments on sensory afferent development is region-specific, being localized to the synaptic neuropil.

Previously, the inhibition of sensory afferent defasciculation was studied after treating cultured germinal plates with threshold

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concentrations of CE0 Fab (3-12 nM) for 36 hr. To shorten the time of treatment necessary for obtaining a perturbation effect, we tested several higher doses of Fab fragments (20, 50 and 100 nM). We studied the kinetics of sensory afferent projection that developed at nearly normal rate in the presence of an intermediate concentration of Fab fragments (50 nM) using improved culture conditions for the germinal plate. We found that the rate at which defasciculated axons are lost can be modeled by a first order decay reaction $y = 54*10^{-0.52x}$ in which the average number of defasciculated axons (y) in a specific time (x) is a simple function of the total axon population. If the rate (y', the derivative of y) at which defasciculated axons are lost as a function of perturbation time (x) is exponentially proportional to the number of axons then: $y' = -18.73* 10^{-0.152x}$. The half life for the survival of defasciculated axons is 1.98 hr, which means that nearly every 2 hr, 50% of the defasciculated axons will disappear.

In the simplest case, a first order decay process has a single rate limiting step. A less likely possibility is that this process involves more than one rate contributing step that occurs at comparable rates. In the hypothetical scheme illustrated in Figure 8, the binding of CE0, the mannose epitope, to its hypothetical carbohydrate-binding protein or lectin, L , and its Fab fragments is governed by two rate constants K^1_{eq} and K^2_{eq}, respectively. The rate limiting step in this scheme is the loss of defasciculated axons, its rate constant being $k_{obs} = 0.152$. However, it is also possible that the binding of CE0 to its lectin constitutes the rate limiting step. Imaging live axons may provide evidence supporting the idea of

axonal loss as rate limiting, if, for example, it could be shown that the average time for the collapse of defasciculated axons into axonal tracts is comparable to the half life computed from our model.

The kinetics of the inhibition of defasciculation also suggest that this process is not limited by diffusion. This finding is consistent with the absence of a connective tissue boundary in the early embryonic nervous system (Thorey & Zipser, 1993).

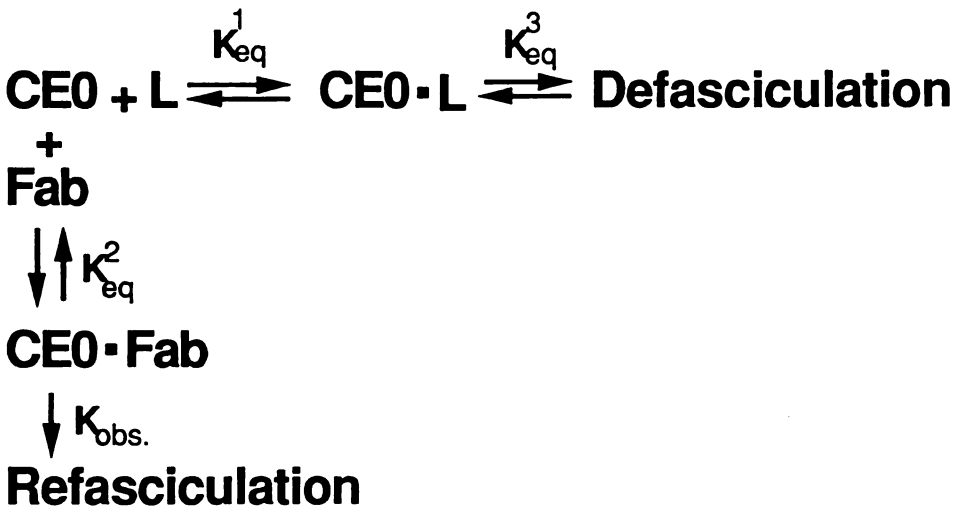
Novel observations on the effects of Lan3-2 Fab fragments on sensory afferent development.

A novel observation was that the susceptibility of axonal defasciculations to Fab fragments is age-dependent. Axons projecting into the neuropil in the presence of Fab fragments are inhibited from defasciculating at all concentrations of Fab fragments tested. Axons that innervated ganglia one day prior the application of Fab fragments are perturbed with lower Fab fragments concentrations than axons that had innervated ganglia two days earlier. The loss of sensitivity to Fab fragments with increasing age correlates with the appearance of the other carbohydrate epitopes on 130 kD glycoproteins. These other epitopes, containing galactose or glucose, mediate the assembly of previously defasciculated axons into defined subregions of the sensory neuropil (Song & Zipser, submitted, c; Song & Zipser, submitted, b). The loss in sensitivity to CE0 Fab fragments also correlates with the onset of expression of CE0 on integral membrane proteins. Axons newly defasciculating into the neuropil express CE0 only on their 130 kD proteins that

behave like loosely associated extrinsic membrane glycoproteins (Bajt, et al., 1990a) which theoretically, may move freely by lateral diffusion on the cell surface, independent of the intensity of the transverse binding between their carbohydrate epitopes and putative carbohydrate-binding proteins. In contrast, the later expression of CE0 by afferents on their 103/95 kD integral membrane protein (McGlade-McCulloh, Muller, & Zipser, 1990) could, by analogy to other systems (Hynes & Lander, 1992), signal the onset of stronger adhesion to their substrate and thus, a decreased sensitivity to perturbations by Fab fragments.

An effect of Lan3-2 Fab fragments that we observed here for the first time is a stimulation in the rate of innervation of segmental ganglia by the median nerve, which as discussed above, indicates an increase in the rate of sensillum 3 differentiation. This effect was only observed in response to a low concentrations of Fab fragments (20 nM). Further work that directly relates the function of CE0 to the differentiation of sensory afferents cell bodies is needed to verify a potential growth promoting role of this mannose-containing epitope.

Figure 8. A model of CE0 carbohydrate interactions and perturbations. In our model, the binding of CE0, the mannose epitope, to its hypothetical lectin, L , and its Fab fragments is governed by two rate constants K^1_{eq} and K^2_{eq}, respectively. The normal interaction between mannose-specific CE0 and its corresponding lectin enables axons to defasciculate into the CNS neuropil, governed by rate constant K^3eq. Adding Fab fragments competes binding of CE0 to lectin. Blocking the interaction between CE0 and lectin by Fab fragments leads to the refasciculation of axons, thereby inhibiting axonal defasciculation. The rate limiting step is the loss of defasciculated axons, its rate constant being $k_{obs} = 0.152$.


CE0 + L
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CHAPTER 2. TARGETING OF AN AXONAL SUBSET MEDIATED BY ITS SEQUENTIALLY EXPRESSED CARBOHYDRATE MARKERS

We are studying the molecular mechanisms directing the targeting of sensory afferents in the embryonic leech. Sensory afferents grow from the periphery to make connections within the segmental ganglia of the CNS. Using molecular perturbations of cultured embryos, we found that mannose- and galactose-containing glycoconjugates have separate functions in the emerging organization of the sensory neurons' target region. A constitutive marker common to all of these neurons mediates the initial dispersal of their axons across the entire target region via mannose-specific recognition (previous chapter). In contrast, a marker expressed later in development by a subset of these neurons restricts their axons to a discrete subregion via galactose-specific recognition. Thus, by performing opposing functions in a temporal sequence, carbohydrate markers collaborate in the targeting of an axonal subset during the development of neuronal connectivity. To our knowledge, this is the first demonstration of a physiological role of cell type-specific surface carbohydrates in neuronal targeting.

INTRODUCTION

Neuronal connections in many sensory systems are formed as the initially diffuse axonal innervation of a target region is sculpted

or structuralized into columns, layers or other subregions. Previously, it was shown that patterned electrical activity leads to the structuralization of target regions and the concomitant refinement of neuronal connectivity (Shatz & Stryker, 1988; Shatz, 1990; Constantine-Paton, Cline, & Debski, 1990). However, the mechanisms that mediate the initial targeting of axons in the synaptic neuropil have remained elusive. Mapping axonal trajectories in vivo and vitro in an attempt to uncover positional clues has led to conflicting information (Nakamura & O'Leary, 1989; Cox, et al., 1990; Simon & O'Leary, 1992)

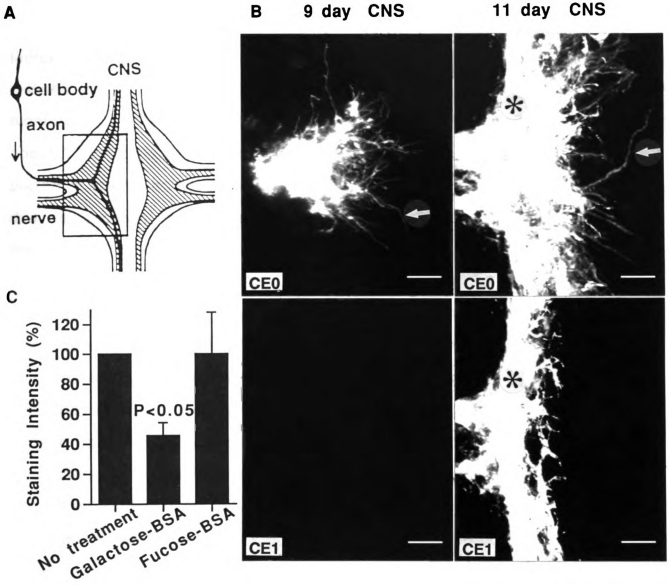
Here we test the idea that surface glycoconjugates which mark functional sets of neurons (Dodd & Jessell, 1985; Whitlock, 1993; Zipser, et al., in press; Peinado, et al., 1990; Riddle, et al., 1993; Scott, et al., 1990) act as recognition molecules helping neurons to choose their target regions where they integrate into synaptic networks. Previously, it has been difficult to demonstrate the importance of specific neuronal carbohydrate markers, presumably because they are expressed on neurons only as long as the nervous system remains intact. Partially deafferented neurons in brain slices or isolated neurons in dissociated culture lose their specific carbohydrate markers and instead express anachronistic surface carbohydrates that can resemble those of undifferentiated neuroblasts (Naegele & Katz, 1990; Dodd & Jessell, 1985; Oudega, et al., 1992; Barakat, et al., 1989). Therefore, we developed the cultured leech embryo as a model system in which the role of normally differentiating glycoconjugates marking functional sets and subsets of neurons can be studied in a virtually intact central

nervous system. We found that two glycoconjugates have different and fundamental impacts on the projections of neuronal processes within their target region.

Like their vertebrate counterparts (Dodd & Jessell, 1985; Riddle, et al., 1993; Scott, et al., 1990), functional sets and subsets of leech sensory neurons are specifically marked with different glycoconjugates (Zipser, et al., 1994; Peinado, et al., 1990). The full set of leech sensory afferent neurons can be identified via a mannose-containing epitope, their generic marker CE0 (Carbohydrate Epitope 0), while discrete subsets of these neurons correlating with different sensory modalities, can be identified via three other carbohydrate epitopes: the subset markers CE1, CE2, and CE3. All of these neuronal markers are located on N-linked carbohydrate chains of 130 kD surface proteins (Bajt, et al., 1990a). Antibodies against any of these carbohydrate markers stain the peripheral cell bodies of sensory neurons and their tightly bundled axons, which project through peripheral nerves into their target regions in the CNS (Figure 9A and B). We used the emerging organization of the sensory neurons' target region as a morphological indicator for the establishment of their precise synaptic connectivity. As we demonstrated in previous studies (Zipser, et al., 1989; Zipser & Cole, 1991), sensory neurons use their mannose-containing marker, CE0, to defasciculate and disperse their axons across the entire target region as they first enter the CNS via a process that can be modeled by a first order decay reaction (Song & Zipser, submitted, a). Here we report that later in development, CE1, a subset marker, has the opposing role of

restricting its respective axonal subset into a discrete subregion of the sensory neurons' target region.

Figure 9. Sequential expression of two different carbohydrate markers on differentiating leech sensory afferent neurons.
A. Diagrammatic representation of the sensory afferent system. The peripheral cell body of a sensory neuron projects its axon into the CNS where it contacts its postsynaptic targets (Fernandez, 1978). Staining the full set of sensory neurons for the carbohydrate epitope CE0, outlines the full extent of the sensory neurons' target region (hatched area). In contrast, staining subsets of these sensory neurons for their carbohydrate markers CE1, CE2, and CE3 defines discrete subregions, the CE1, CE2 and CE3 target regions (not shown here) (Peinado, Macagno, & Zipser, 1987; Peinado, Macagno, & Zipser, 1987; Zipser, Erhardt, Song, Cole, & Zipser, 1994b).
B. The sequential expression of carbohydrate markers is illustrated by double-labeling 9-day and 11-day embryos for both the full-set marker, CE0, and the subset marker, CE1, using direct immunofluorescence. Axons displaying these carbohydrate markers are illustrated in optical sections through the left half of the CNS (boxed area in Figure 1A). Both top panels: By embryonic day 9, CE0 axons enter the CNS, defasciculate and disperse as single axons across the target region (arrow). By embryonic day 11, CE0 axons occupy the target region more extensively. Some of the CE0 axons are now densely grouped (asterisk), while others are still loosely dispersed as single axons (arrow). Both bottom panels: By day 11, CE1 is expressed on those axons that have become densely grouped into a subregion (asterisk). However, none of the dispersed axons, identified on day 9 and 11 by CE0, expresses CE1. The temporal sequence of expression of CE0 and CE1 was verified in 90 embryos. Bar, 5 μm.
C. The chemical composition of the developmentally regulated CE1 was characterized by competing the binding of its respective monoclonal antibody (CE1 antibody) with different carbohydrates (n = 6). Embryos were briefly treated with just CE1 antibody or with CE1 antibody that had been preincubated with 50 μM galactose-BSA or 50 μM fucose-BSA. The presence of galactose-BSA decreased the staining of CE1 axons by more than 50%. Fucose-BSA did not have a significant effect on the staining of CE1 axons.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MATERIALS AND METHODS

Specimen preparation

Leeches of the *Hirudo medicinalis* species were bred at room temperature in artificial seawater (Forty Fathoms; 0.5 gm/liter distilled water), and fed with cow blood. After isolation for 2 months at 15 ^{0}C, adult leeches were brought together for mating for about 1 month under room temperature. The gravid leeches were then placed in plastic boxes containing moist sphagnum moss and checked daily for cocoons. Once found (day 0), the cocoon was transferred to the incubator at 20^{o}C.

Embryos of a desired age were removed from their cocoons which typically contains between 12 to 18 sibling embryos of the same developmental age. The epithelial envelope of the embryos was then opened by a dorsal incision to remove the yolk, and the germinal plate was exposed. The germinal plates of embryos of a given cocoon were divided into different control and experimental categories. Each category contains at least three siblings. The germinal plates were then stretched out by pinning their epithelial envelopes on UV-sterilized Sylgard-coated culture dishes (35 x10 mm) (Corning Glass Works. Corning, NY), followed by washing three times with sterile tissue culture medium. The germinal plates belonging to the same category were cultured for various periods of time at 20^{0}C in the same dish in 1 ml of the enriched growth medium consisting of Leibovitz-15 (Gibco laboratories, Grand Island, N.Y) with 1% ITS^{+}(Collaborative Research Inc., Bedford, MA), 1nM nerve growth

factor (NGF) (Sigma, St. Louis, MO), 10 nM epidermal growth factor (EGF) (Collaborative Res. Inc), and 10 mM potassium. To determine the developmental stage of sensory afferent differentiation, germinal plates were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at the onset of culturing and then stained using indirect immunoperoxidase methods described by Zipser and Cole (1991).

Monoclonal antibodies and their Fab fragments

Monoclonal antibodies (mAbs) were generated in this laboratory either against homogenized leech CNS (Zipser & McKay, 1981) or against excised gel bands with 130 kD proteins extracted from leech CNS (Flaster, Schley, & Zipser, 1983). MAbs Laz2-369 and Laz7-79 are directed against carbohydrate epitopes (CE1 and CE2 respectively) on the surface of sensory afferent neurons. For double-labeling, mAbs were biotinylated following methods described by Harlow & Lane (1988). For live staining, mAbs were conjugated to 5-carboxytetramethylrhodamine (RITC) or 5-carboxyfluorescein, succinimidyl ester (FITC) (Molecular Probes, OR) following methods of Harlow and Lane (1988).

Monovalent Fab fragments rather than whole IgGs were used in the perturbation experiments, because the two antigen binding sites of a whole IgG can crosslink membrane surface molecules, eliciting perturbation effects by grossly distorting membrane topology. The Fab fragments of these mAbs were prepared from the ascites fluid according to the description by Harlow & Lane (1988). Ascites fluid

was precipitated with 40% saturated ammonium sulfate with gentle shaking on ice for 24 hrs, followed by dialysis against PBS/4 mM EDTA at 4°C for 36 hrs. Then the dialyzed ascites was digested with mercuripapain (Worthington Biochemical Co., Freehold, NJ), followed by dialysis again against 5mM Tris-HCl buffer (pH8.0) overnight at 4°C. MAb ascites was then applied to a 2.5 ml DEAE cellulose (Whatman Bio. Ltd) column with gradient wash solution of 0 to 0.3M NaCl. The concentration of Fab fragments per fraction was estimated assuming that 1mg/ml of immunoglobulin has an absorption of 1.4 at 280 nm, measured by spectrophotometer (DU series 62, Beckman). As determined on silver-stained sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), Fabs have a molecular weight of 55 kDa (Harlow & Lane, 1988).

Immunocytochemistry

Antibody staining of fixed specimen: germinal plates were washed with 0.9% NaCl, 50 mM phosphate buffer (PBS, pH 7.4), and fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 30 min at room temperature. Embryos were post-fixed with absolute methanol for 15 min followed by extraction in absolute xylene for 5 min. The specimens were rehydrated with absolute methanol, 70% ethanol, and PBS. Antibodies and avidins were diluted with PBS/3% bovine serum albumin (Sigma) /2% Triton X-100 (Sigma). All except the final rinsing step during the antibody staining procedures were carried out with the same buffer. The final rinse was performed with PBS. For staining sensory afferents with a single mAb, germinal plates were

incubated overnight with hybridoma supernatant (1/20). After rinsing, embryos were incubated for 3 hr with biotinylated $(Fab')_2$ (1/100; Dako Corp., Carpenteria, CA). For double labeling sensory afferents with two different mAbs, germinal plates were first incubated overnight with the CE0 mAb (ascites fluid, 1/3000). After rinsing, the specimen were incubated for 2 hr with rhodamine-conjugated rabbit anti-mouse IgG (1/50) (Dako Corp). After rinsing, the specimens were treated with ascites fluid of another mouse mAb (1/10%) to block anti-mouse IgGs. Next, the germinal plates were incubated for 4 hr in biotinylated CE1 mAb (1/200). After rinsing, they were treated for 1 hr with avidin-FITC (1/600) (Vector Lab., Burlingame, CA).

Live sensory afferents were stained by incubating freshly dissected germinal plates for 3 hr in L-15 to which fluorescently-conjugated mAbs were added. The full set of neurons was stained for its CE0 epitope with Lan3-2-RITC (1/100); the large subset was stained for its CE1 epitope with Laz2-369-FITC (1/20).

The fluorescently-stained embryos were mounted with 70% glycerol to which saturating levels of p-phenylenediamine (Sigma; Cat. # P-1519) had been added to prevent fading of fluorescence.

Characterization of Carbohydrate marker CE1

Fluorescein-conjugated CE1 mAb was diluted (1/40) into L15 (no supplements) and different aliquots were preincubated for 12 hr with 50 μM of different neoglycoproteins (see below). Freshly dissected germinal plates were treated for 3 hr with just CE1 mAb

or with CE1 mAb that had been preincubated with different neoglycoproteins. The germinal plates were then rinsed, fixed in 4% paraformaldehyde, and embedded in 70 % glycerol to which p-phenylenediamine was added to prevent bleaching of fluorescence. The binding of mAbs to CE1 antigen was measured by assaying the intensity of sensory afferent staining in the different embryos under the confocal microscope.

Perturbation experiments

The perturbation experiments were performed on germinal plates that were conditioned to culturing by a 6 hr preincubation period, because no significant axonal growth occurs during the first 6 hr of culture (Song & Zipser, submitted, a). The sibling embryos of an 11 day old cocoon were divided into several groups that were cultured for an additional 6 hr in the absence of reagents (no treatment) or in the presence of Fab fragments (40 nM), neoglycoproteins (4 μM, Sigma), or enzymes (20 units/mL, Sigma). After rinsing, the embryos were fixed and sensory afferent projections were stained with either CE0 or CE1 mAb. The sensory afferent projections were analyzed under the Odyssey, a laser scanning confocal microscope (Noran, Madison, WI).

Data analysis of immunofluorescently stained tissue by the confocal microscope

The fluorescent stained projections of sensory afferents were analyzed under the Odyssey Real Time Laser Scanning confocal microscope (Noran instrument, Inc; Madison, WI) equipped with Image-1 Software (Universal Imaging Corp., PA), which allows us to capture serial images of sections to reconstruct the three-dimensional images and analyse the features of images. For fluorescein, the excitation and primary barrier filters were 488 and 515 nM, respectively. For rhodamine, the excitation and primary barrier filters were 529 and 550, respectively. A 100 x oil immersion objective (1.44 NA) was used for capturing images.

The ipsilateral neuropil of the embryonic CNS was optically sectioned in 1 μm increments. Axonal projections of sensory neurons were illustrated as reconstructed images that were formed by overlaying 12 serial optical sections. Optical sections of CNS devoid of fluorescently stained axons were captured ventral to the target region of sensory neurons and subtracted as background from the reconstructed images using the function "Graphic function: Logic Function" of Image-1. The background substracted reconstructed images were used to analyze the blocking of antibody binding to CE1 axons and the perturbation of CE1 axons with Fab fragments.

The blocking of antibody binding to CE1 axons with neoglycoproteins was quantified using the function "Brightness Measurement: Area Brightness" of Image-1. The intensity of fluorescently stained CE1 axons was measured in their neuropil

target region within a 10 x 20 μm area (medial-lateral x anterior-posterior) corresponding to the boxed area in Figure 1.

To quantify the perturbation results, we performed the function of Image-1 called "Brightness Measurement: Line Intensity Scan". To measure CE1 axons leaving their target region by crossing its medial border, we positioned a scanning line parallel to the medial border of the CE1 target region, but displaced by 5 μm (Figure 10). The scanning line was 232 pixels long (20 μm) and 1 pixel wide. The pixel value due to the fluorescent intensity of all the stained axons crossing the scanning line was measured. Furthermore, the average pixel value due to a single axon crossing the scanning line was measured. To estimate the total number of CE1 axons leaving their target region, the pixel value of the entire scanning line was divided by the average pixel value of a single fluorescent axon.

All the statistics were performed by Student T test (two tails).

Experiment reagents

The glycosidases and neoglycoproteins were purchased from Sigma, MO. β-N-Acetylglucosaminidase (Cat. # A2264); Cellulase (Cat. # C2415); β-Glucosidase (Cat. # G0395); α-Glucosidase (Cat. # G6136); β-Galactosidase (Cat. # G6512); α-Galactosidase (Cat. # G6762); α-Mannosidase (Cat. # M1266). Galactose-BSA (Albumin, Bovine-Galactosamide; 15-25 mol monosaccharide/mol albumin; Cat. # A5908); Fucose-BSA (Albumin, Bovine-Fucosylamide; 15-25 mol monosaccharide/mol albumin; Cat. # A6033), Cellobiosyl-BSA (Albumin, Bovine-Cellobiosyl; 15-25 mol disaccharide/mol albumin;

Cat. # A5408), Mannose-BSA (Albumin, Bovine-p-Aminophenyl-α-D-Mannopyranoside; 20-30 mol monosaccharide/mol BSA; Cat#. A4664), N-Acetyl-Glucosamine-BSA (Albumin, Bovine-p-Aminophenyl-N-Acetyl-β-D-Glucosaminide; 15-25 mol monosaccharide/mol albumin; Cat. # A1034), Glucose-BSA (Albumin, Bovine-Glucosamide; 15-30 mol monosaccharide/mol albumin; Cat. # A 6158).

RESULTS

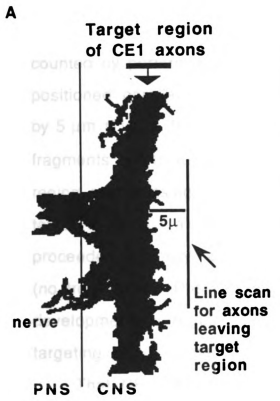
The sequential expression of carbohydrate markers by differentiating leech neurons is found by double-labeling embryos for both the full-set marker, CE0, and the subset marker, CE1, using direct immunofluorescence. Axons displaying these carbohydrate markers are illustrated in optical sections through the left half of an embryonic leech CNS (boxed area in Figure 9A). Because of its constitutive expression, CE0 serves as a marker for sensory neurons that track through peripheral nerves in a tight bundle and then, upon entering the CNS, defasciculate in a local region to disperse as single axons across the entire sensory neurons' target region (Figure 9B; 9 day top panel, arrow). Proliferating sensory neurons continue to invade the CNS and therefore populate the target region more extensively by embryonic day 11 (Figure 9B; 11 day top panel). Some of these CE0 axons are now densely grouped (asterisk), while others are still loosely dispersed as single axons (arrow). By day 11, CE1 is expressed on those axons that become densely grouped into a discrete subregion within the ganglion, the CE1 target region (Figure 9B; 11 day bottom panel, asterisk). However, none of the dispersed axons, identified on day 9 and 11 by CE0 (Figure 9B, arrows in both top panels), express CE1 (both bottom panels). Thus, we find that the transition of axonal projections from a diffuse to a grouped pattern in the target region is accompanied by the regulated onset of expression of CE1.

The chemical composition of CE1, the developmentally regulated subset marker, was characterized by competing the

binding of its respective monoclonal antibody (CE1 antibody) with different carbohydrates. Embryos were briefly treated with only CE1 antibody or with CE1 antibody that had been preincubated with neoglycoproteins which are monosaccharides linked multivalently to a carrier protein such as BSA. Only the presence of galactose-BSA (50 μM) significantly reduced the staining of CE1 axons (<50 %) (Figure 9C), but not the presence of fucose-BSA (50 μM), or (not shown here) cellobiosyl-BSA and mannose-BSA. That is, the galactose-BSA bound to the CE1 antibody, thereby preventing it from binding to CE1 on the surface of axons. This indicates that CE1, the later expressed subset marker, contains galactose, in contrast to CE0, the constitutive marker, which contains mannose (Bajt, et al., 1990a; McKay, et al., 1983).

To investigate the developmental role of CE1, the structurally intact nervous system of leech embryos was experimentally manipulated with very low concentrations of Fab fragments, neoglycoproteins and enzymes during a six-hour incubation period in defined enriched growth medium. When cultured in growth medium alone, CE1 axons normally assemble in their target region (Figure 10A; 10B; no Fab). However, the addition of CE1 Fab fragments (40 nM) led to a massive scattering of axons away from the CE1 target region (Figure 10B, arrow; CE1 Fab). As a control for the specificity of this perturbation effect, sibling embryos were treated with CE2 Fab fragments (40 nM) directed to the carbohydrate marker of the CE2 subset which occupy the CE2 target region (Peinado, et al., 1987; Zipser, et al., 1994b). The targeting of CE1 axons was not perturbed by CE2 Fab fragments

Figure 10. Targeting of an axonal subset mediated by its developmentally regulated carbohydrate marker.
A. Schematic representation of the assembly of CE1 axons into the CE1 target region. Also shown is the position of the scanning line, parallel to the CE1 target region, but displaced medially by 5 μm, along which a line scan was performed to assess the perturbations of the CE1 axons.
B. Optical sections illustrating CE1 axons in sibling embryos that were cultured either in defined growth medium (top panel; no Fab), or in the presence of 40 nM CE1 Fab fragments (middle panel; CE1 Fab), or in the presence of 40 nM CE2 Fab fragments (bottom panel; CE2 Fab). In the embryo cultured in the absence of Fab fragments, targeting of CE1 axons proceeded normally. In contrast, in the sibling embryo treated with CE1 Fab fragments, CE1 axons were massively scattered across the scanning line (arrow). The targeting of CE1 axons was not perturbed in the embryo that was treated with CE2 Fab fragments. Bar, 5 μm.


A
Target region
of CE1 axons
5μ
Line scan
for axons
leaving
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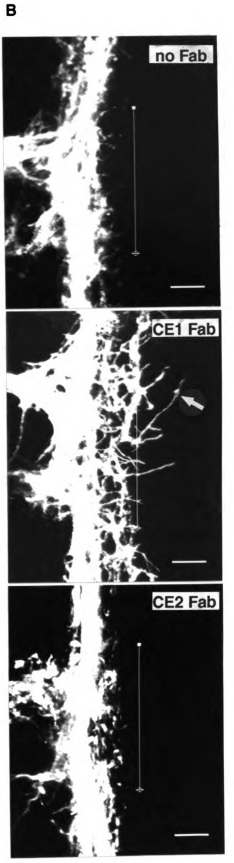
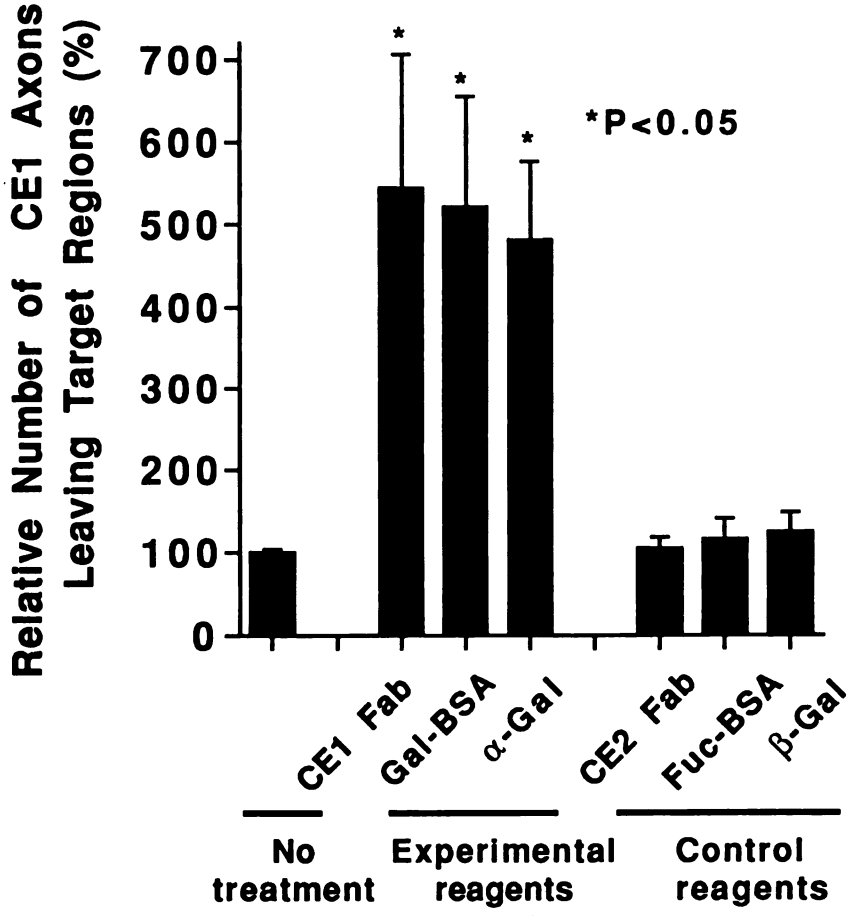
region
nerve
PNS
CNS


B
no Fab
CE1 Fab
CE2 Fab

(Figure 10B; CE2 Fab). The number of CE1 axons leaving their target region under the different experimental and control conditions was counted by performing a line scan along a scanning line that was positioned parallel to the CE1 target region, but medially displaced by 5 μm (Figure 10A). The treatment of embryos with CE1 Fab fragments led to a five-fold increase in axons leaving the CE1 target region and crossing the scanning line (Figure 11). In contrast, the targeting of CE1 axons in embryos treated with CE2 Fab fragments proceeded normally, as in embryos cultured without Fab fragments (no treatment). This is the first line of evidence that CE1, the developmentally regulated carbohydrate marker, mediates the targeting of its respective axonal subset.

That CE1, the galactose-containing subset marker, mediates the targeting of CE1 axons was supported by two other independent experimental manipulations. Competition studies with galactose-BSA mimicked the perturbation effect of CE1 Fab fragments (Figure 11). A five-fold increase of axons leaving the CE1 target region was observed in embryos that were cultured in the presence of galactose-BSA (4 μM). In contrast, the presence of fucose-BSA (4 μM) did not interfere with the normal targeting of CE1 axons. A similar five-fold increase in axons leaving the CE1 target region was also elicited by modifying endogeneous galactose structures with α-galactosidase (20 units/ml). In contrast, β-galactosidase (20 units/ml), as well as mannosidase, cellulase and glucosidase (which are not shown), had no significant effects. These observations suggest that the targeting of CE1 axons is mediated by

Figure 11. Quantification of the perturbation of axonal targeting by Fab fragments, neoglycoproteins and exoglycosidases. For the antibody perturbation experiments (n = 8), sibling embryos were treated with CE1 and CE2 Fab fragments as described in Figure 2. For the neoglycoprotein perturbations (n = 7) , sibling embryos were treated with galactose-BSA (4 μM) or fucose-BSA (4 μM). For the enzyme perturbations (n = 7), sibling embryos were treated with α-galactosidase (20 units/ml) or β-galactosidase (20 units/ml). Each experiment included a group of 3 sibling embryos that were cultured in the absence of experimental and control reagents (no treatment). The success or failure of the targeting of CE1 axons under the different conditions was quantified by performing a line scan (see diagram in Figure 2A) on optically sectioned embryonic CNS. Culturing embryos in the presence of the experimental reagents, CE1 Fab fragments, galactose-BSA or α-galactosidase, led to a five-fold increase in the number of CE1 axons leaving their target region. All values were set relative to the value for embryos cultured in defined medium (no treatment). Culturing sibling embryos in the presence of control reagents, CE2 Fab fragments, fucose-BSA, or β-galactosidase, did not significantly perturb the targeting of CE1 axons. The treatment with glucose-BSA and mannose-BSA (4 μM each) also did not increase the number of CE1 axons leaving the CE1 target region (not shown).


Relative Number of CE1 Axons Leaving Target Regions (%)
700
600
500
400
300
200
100
0
*
*
*
*P<0.05
CE1 Fab
Gal-BSA
α-Gal
CE2 Fab
Fuc-BSA
β-Gal
No treatment
Experimental reagents
Control reagents

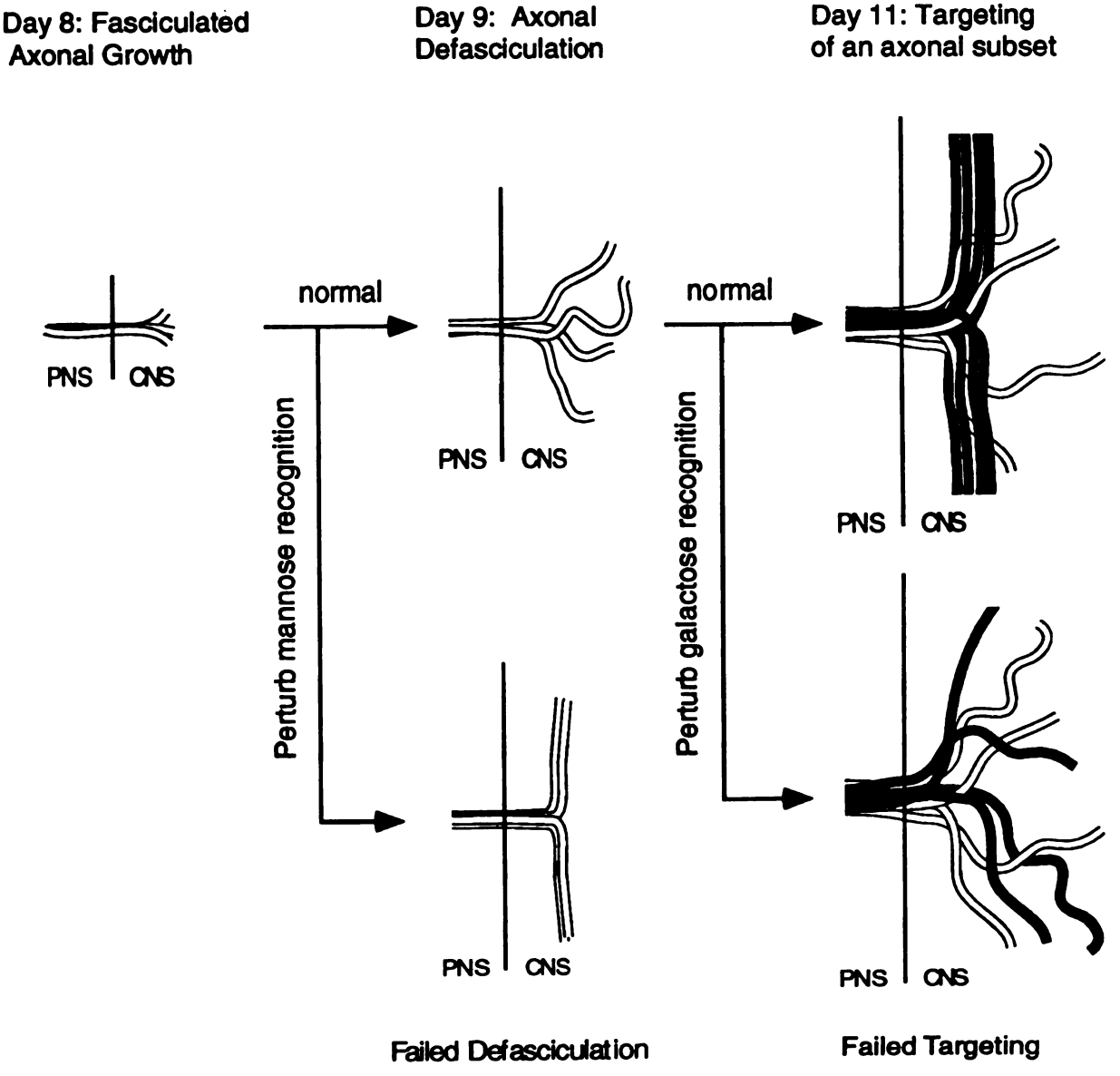
endogeneous α–extended galactose structures. There may be a phylogenetic conservation of this type of carbohydrate recognition event because α–extended oligosaccharides are also markers for subsets of mammalian sensory neurons (Jessell & Dodd, 1985).

DISCUSSION

We have combined cellular and molecular approaches for studying neuronal connectivity using the embryonic leech as a physiological system. Culturing the intact germinal plate of the leech embryo in defined enriched growth medium permits sensory afferent neurons to differentiate normally. After having projected through peripheral nerves in tightly bundled axon tracts, sensory afferents defasciculate and diffusely innervate their target region, the sensory neuropil of the CNS ganglia. Previously, we showed that this primary targeting step is mediated by a mannose-containing epitope on their cell surface via a process that can be modeled as a first order decay reaction (Zipser, et al., 1989; Zipser & Cole, 1991; Song & Zipser, submitted, a). Subsequently, as we demonstrated here, a subset of these axons begins to express a galactose-containing epitope and concomitantly, assembles itself into a defined subregion of the sensory neuropil. This secondary targeting step is mediated by the galactose-epitope that serves as a specific marker for the axonal subset. Thus, in this two-step process, the transition from a diffuse to a structured pattern is mediated by sequentially expressed carbohydrate markers. Figure 12 summarizes schematically these two separate carbohydrate, that, by performing opposing functions in a temporal sequence, collaborate in the targeting of an axonal subset during the development of neuronal connectivity.

Our finding that the transformation from diffuse to structured projection occurs as a result of newly expressed surface

Figure 12. Diagrammatic representation of sequential carbohydrate recognition steps leading to axonal targeting. Newly born sensory afferents expressing their constitutive mannose-containing marker, CE0, track through the peripheral nerves (PNS) in a tightly bundled formation on embryonic day 8. These axons enter the CNS on embryonic day 9, and defasciculate into their target region via a mannose-specific recognition involving CE0. Inhibition of the mannose-specific recognition leads to a failed defasciculation of all axons. Second, after a developmental delay, on embryonic day 11, an axonal subset begins to express its galactose-containing marker, CE1 (black axons). This axonal subset now targets a discrete subregion via a galactose-specific recognition involving CE1. Other axons (white) that are newly arriving in the CNS and therefore lack a subset marker are still dispersed. Inhibition of galactose-specific recognition results in failed targeting of CE1 axons. Thus, by performing opposing functions in a temporal sequence, carbohydrate markers collaborate in the targeting of an axonal subset during the development of neuronal connectivity.


Day 8: Fasciculated Axonal Growth
Day 9: Axonal Defasciculation
Day 11: Targeting of an axonal subset
PNS
CNS
normal
Perturb mannose recognition
normal
Perturb galactose recognition
Failed Defasciculation
Failed Targeting

carbohydrate markers may be pertinent to other systems. Mapping the projections of axons into their target regions also has been a popular approach for exploring mechanisms of neuronal connectivity in the retinotectal system (Holt & Harris, 1993). However, in vivo measurements so far have established that the initial projections of developing or regenerating retinal axons into the tectum are a function of the size of the tectum (Holt & Harris, 1993; Nakamura & O'Leary, 1989; Simon & O'Leary, 1992). If the tectum is already large, retinal axons make long, overshooting projections before they begin generate interstitial branches in appropriate subregions. If a tectum is small during its initial innervation, axonal projections at first demonstrate substantial overlap before they subsequently become segregrated into subregions as the tectum undergoes its substantial expansion.

In contrast, in vitro measurements have provided evidence that a subset of retinal axons is subject to powerful guidance by positional activity in the tectum (Cox, et al., 1990; Simon & O'Leary, 1992). However, the physiological significance of this observation is not clear because axons from the same system behave differently in vivo and in vitro (Nakamura & O'Leary, 1989; Simon & O'Leary, 1992). Because in the leech system, sensory afferents behave the same in vivo and in the intact-cultured germinal plates, the in vitro perturbation experiments reported here provide evidence on molecular mechanisms of axonal targeting that is of direct physiological relevance.

In leech embryogenesis, the initial target region innervated by the first sensory afferents is very small because the ipsilateral

neuropil of the segmental CNS ganglion is only 25 μm in diameter at that stage (appropriate segmental ganglia in 9 to 11 day embryos). The earliest observation of specific targeting in a particular segmental ganglion is made two days later after its neuropil has grown to a larger size. At this stage, a subset of axons has begun to express the α-galactose extended epitope CE1 (carbohydrate epitope 1). Interestingly, CE1 axons assemble into a subregion of the sensory neuropil that is immediately adjacent to where the first sensory afferents entered. In contrast, other axons devoid of the subset epitope project beyond the CE1 subregion into the more medial aspect of the expanding sensory neuropil. Axons projecting beyond the CE1 subregion demonstrate the same diffuse defasciculated growth as the first axons that entered two days earlier. These diffusely projecting axons lacking CE1 pioneer the medial tracts closer to the midline of the neuropil (Song, unpublished). Thus, the leech sensory afferents resemble frog retinotectal axons in their initial overlapping projection into a small target region and the later segregation of axons into five distinct subregions of the sensory neuropil as the target region enlarges (Song & Zipser, submitted, b; Zipser, et al., 1994).

The signal leading to the induction of the galactose epitope on axons that successfully assemble into their subregion in the sensory neuropil is not known. Because sensory neurons express their subset markers only after a two-day sojourn in the CNS, the signal regulating their oligosaccharide processing may emanate from other neurons that they contact in the sensory neuropil, some of whom become their future postsynaptic partners. In other systems there

are examples of target structures modifying the phenotype of presynaptic neurons (Habecker & Landis, 1994; Nawa, Yamamori, Le, & Patterson, 1990).

Our three separate lines of experimental evidence indicate that the assembly of sensory afferents into their target region is mediated by a galactose-specific recognition. While the ligand in this interaction is the galactose-containing epitope on the axonal surface, the nature of its receptor has yet to be explored. In classical carbohydrate recognition, carbohydrates bind to carbohydrate-binding proteins or lectins. A possible candidate is the leech galectin that is weakly expressed by central neurons (Zipser, unpublished). Earlier we reported on the strong expression of leech galectin by a small subset of sensory afferents that occupy a special target region, the midline tract. Thus, in the leech, as in vertebrates, galectin is expressed in different concentrations on sensory neurons and their potential postsynaptic partners (Regan, Dodd, Barondes, & Jessell, 1986; Cole & Zipser, 1994a; Cole & Zipser, 1994b). Another form of carbohydrate recognition may involve the homotypic interactions between carbohydrates themselves (Misevic & Burger, 1993; Hakamori, 1992). Interaction among the CE1 subset via its shared galactose-containing epitope would explain the high specificity found in peripheral nerve bundling and target region assembly.

During the early targeting steps, the CE0 and CE1 epitope are located on 130 kD proteins that behave like loosely associated extrinsic membrane glycoproteins (Bajt, et al., 1990a; McGlade-McCulloh, et al., 1990). Phase-separation of leech proteins with

Triton X-114, partitions the 130 kD proteins into the aequous phase. Moreover, large amounts of 130 kD proteins were detected in the supernatant after homogenizing leech CNS in hypotonic buffer. During late embryogenesis, CE0 also appears on 103 and 95 kD proteins which, unlike the 130 kD proteins, behave as integral membrane proteins. Thus, the 130 kD glycoprotein expressed during axonal targeting, theoretically, may move freely by lateral diffusion on the cell surface, independent of the intensity of the transverse binding between their carbohydrate epitopes and putative carbohydrate-binding proteins. It is not known whether the mannose and galactose-containing epitopes are located on the same or different 130 kD proteins. The high degree of cross reactivity of CE1 antibody with 130 kD protein immunoprecipitated by CE0 antibody suggests that the CE1 and CE0 epitopes may be located on the same protein core. However, it appears that CE0 and CE1 can be located on different carbohydrate chains because their respective antibodies recognize different peptide fragments generated from 130 kD protein by limited proteolysis (Bajt, et al., 1990a).

It is interesting that CE0, which is constitutively expressed by sensory afferents, contains mannose while CE1, which is expressed later by an axonal subset, contains galactose. Mannose is a carbohydrate residue that is characteristic of an early step in the processing of N-linked oligosaccharide chains, while galactose is a residue that is characteristic of hybrid or complex type carbohydrates generated during a later stage of oligosaccharide processing (Kornfeld & Kornfeld, 1985). In sensory afferent targeting, the galactose-specific recognition appears to be stronger

than the mannoses-specific recognition. CE1 axons that express the galactose-containing epitope are no longer perturbed by reagents that disrupt mannose-specific recognition. They are only perturbed by reagents that disrupt galactose-specific recognition.

Carbohydrates on the neuronal surface or in the matrix are now being associated with many different developmental events involving the regulation of cell adhesions or recognition. Highly acidic carbohydrates, such as sulfated proteoglycan, exercise a repulsive effect by creating inhibitory boundaries to axonal growth (Brittis, Canning, & Silver, 1992). However, acidic carbohydrate may also promote neuronal migration, axonal extension or branching (Grumet, Flaccus, & Margolis, 1993; Wang & Denburg, 1992; Streit, Nolte, Rasony, & Schachner, 1993), perhaps by counteracting other interactions that may provide too strong an adhesion and thus inhibit movement. One example is the L2/HNK-1 epitope (Chou, Ilyas, Evans, Costello, Quarles, & Jungawala, 1986; Chou, Prasadarao, Koul, & Jungalwala, 1991; Chou, et al., 1991; Bajt, et al., 1990b) promoting the migration of neural crest cells (Bonner-Fraser, 1985; Hall, Liu, Schachner, & Schmitz, 1993; Künemund, Jungalwala, Fischer, Chou, Keilhauer, & Schachner, 1988; Martini, Xin, Schmitz, & Schachner, 1992). Another example is the polysialic acid epitope of NCAM (Rutishauser, Acheson, Hall, Mann, & Sunshine, 1988) promoting the branching of motor axons (Landmesser, Dahm, Tang, & Rutishauser, 1990). Likewise, mannose-containing epitopes appear to play a role in neuronal migration (Lehmann, Kuchler, Theveniau, & Vincendon, 1990). Furthermore, there is a strong case for a growth promoting role of the two oligomannosidic epitopes L3 and L4 (Horstkorte,

Schachner, Magyar, Vorherr, & Schmitz, 1993) on rat neuronal glycoproteins that are evolutionarily conserved and are expressed also on leech 130 kD protein (Bajt, et al., 1990b).

The functions of other carbohydrate residues such as galactose and its derivatives as well as N-acetylglucosamine were less well established. Because of their cell-type expression on different functional classes of neurons (Dodd & Jessell, 1985; Riddle, et al., 1993; Song & Zipser, submitted, b; Song & Zipser, submitted, c; Zipser, et al., 1994; Streit, Schulte, Balentine, & Spicer, 1985; Kivelä, 1992; Baird, Schuff, & Bancroft, 1993; Scott, et al., 1990); they were postulated to play a role in pathfinding or targeting. Also the highly selective distribution of N-Acetylgalactosamine at the neuromuscular junction was consistent with their role in specifying connectivity (Sanes & Cheney, 1982; Scott, Francis, & Sanes, 1988). The glycoprotein associated with the positional activity steering retinal axons in vitro contains galactose residues (Stahl, Mueller, von Boxberg, Cox, & Bonhoeffer, 1990). The experimental evidence provided here is, to our knowledge, the first demonstration of a physiological role of such carbohydrates in neuronal targeting.

CHAPTER 3. STRUCTURALIZATION OF A CNS TARGET REGION MEDIATED BY CARBOHYDRATE MARKERS SPECIFIC FOR NEURONAL SUBSETS

During leech embryogenesis, generic sensory neurons grow as tight axon bundles from the periphery into the CNS where they defasciculate and disperse via a mannose-specific recognition (Zipser & Cole, 1991). Two days after entering the synaptic neuropil, functional subsets of these neurons assemble in stereotypic target regions through different carbohydrate interactions involving newly expressed unique carbohydrate markers. Culturing the intact-germinal plate of the leech embryo in Fab fragments recognizing two subsets of sensory neurons, demonstrated that the targeting response of each subset is separately regulated by their respective carbohydrate markers, CE1 and CE2. Examining the nature of the carbohydrates involved with enzyme or neoglycoprotein treatment indicated that the targeting response of the CE1 subset involves a galactose-specific recognition. In contrast, the targeting response of the CE2 subset involves glucose-specific recognition with a minor galactose component. The nature of the carbohydrate interactions of the two subsets is consistent with their convergent and divergent projections across different target regions.

INTRODUCTION

Sensory information is commonly channeled into multiple target regions in the central nervous system. Some of these target

regions are exclusively innervated by just one sensory modality, while other target regions are multimodal, receiving more than one type of sensory input (Knudson & Brainard, 1991; Draeger & Hubel, 1975; Newmann & Hartline, 1981). Frequently, neurons conveying these different sensory modalities are chemically encoded with different surface glycoconjugates, examples of which are olfactory, mechanosensory and visual neurons (Key & Akeson, 1991; Dodd & Jessell, 1985; Jessell, et al., 1990; Peinado, et al., 1987; Zipser, et al., 1994). The question arises as to what extent, if any, these function-specific carbohydrate markers mediate the targeting of axons during the genesis of neuronal connectivity. The morphological correlate of neuronal connectivity is the structuralization of the synaptic neuropil into layers, columns or other subregions. Identifying the relative contributions of the different neuronal carbohydrate markers in neuropil structuralization requires experimentally manipulating the formation of an intact nervous system, because there is increasing evidence that these carbohydrate markers are lost in neurons deprived of their normal connections (Scott, 1993; Dodd & Jessell, 1985; Oudega, et al., 1992; Barakat, et al., 1989).

Using the intact-cultured germinal plate of the embryonic leech as a model system, we previously demonstrated the involvement of function-specific carbohydrate markers in axonal targeting (Zipser & Cole, 1991; Zipser, et al., 1989; Song & Zipser, 1993). A mannose-containing marker that is constitutively expressed by the entire class of sensory neurons mediates an early step in their targeting response by permitting them to enter and diffusely disperse across

the neuropil. During their initial dispersal across the neuropil, sensory axons lack those carbohydrate markers that will later divide them into modality-specific subsets, the putative mechano-, chemo-, and heatdetectors. After a developmental delay, a subset of these generic sensory neurons begins to express an α–extended galactose epitope, the putative mechanodetector marker. Via a galactose-specific recognition, this subset now assembles into a restricted target region within the sensory neuropil. Here we are contrasting the molecular mechanism by which two different axonal subsets, putative mechano- and chemodetectors, differentially innervate several subregions within the sensory neuropil. We present evidence that the nature of the carbohydrate recognition events mediating the targeting of the two neuronal subsets is commensurate with their convergent and divergent projections into the different subregions of the sensory neuropil.

MATERIALS AND METHODS

Specimen preparation

Leeches of the *Hirudo medicinalis* species were bred at room temperature in artificial seawater (Forty Fathoms; 0.5 gm/liter distilled water), and fed with cow blood. After isolation for 2 months at 15 ^{0}C, adult leeches were brought together for mating for about 1 month under room temperature. The gravid leeches were then placed in plastic boxes containing moist sphagnum moss and checked daily for cocoons. Once found (day 0), the cocoon was transferred to the incubator at 20^{o}C.

Embryos of a desired age were removed from their cocoons which typically contains between 12 to 18 sibling embryos of the same developmental age. The epithelial envelope of the embryos was then opened by a dorsal incision to remove the yolk, and the germinal plate was exposed. The germinal plates of embryos of a given cocoon were divided into different control and experimental categories. Each category contains at least three siblings. The germinal plates were then stretched out by pinning their epithelial envelopes on UV-sterilized Sylgard-coated culture dishes (35 x10 mm) (Corning Glass Works. Corning, NY), followed by washing three times with sterile tissue culture medium. The germinal plates belonging to the same category were cultured for various periods of time at 20^{0}C in the same dish in 1 ml of the enriched growth medium consisting of Leibovitz-15 (Gibco laboratories, Grand Island, N.Y) with 1% ITS^{+}(Collaborative Research Inc., Bedford, MA), 1nM nerve growth

factor (NGF) (Sigma, St. Louis, MO), 10 nM epidermal growth factor (EGF) (Collaborative Res. Inc), and 10 mM potassium. To determine the developmental stage of sensory afferent differentiation, germinal plates were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at the onset of culturing and then stained using indirect immunoperoxidase methods described by Zipser and Cole (1991).

Monoclonal antibodies and their Fab fragments

Monoclonal antibodies (mAbs) were generated in this laboratory either against homogenized leech CNS (Zipser & McKay, 1981) or against excised gel bands with 130 kD proteins extracted from leech CNS (Flaster, Schley, & Zipser, 1983). MAbs Laz2-369 and Laz7-79 are directed against carbohydrate epitopes (CE1 and CE2 respectively) on the surface of sensory afferent neurons. For double-labeling, mAbs were biotinylated following methods described by Harlow & Lane (1988). For live staining, mAbs were conjugated to 5-carboxytetramethylrhodamine (RITC) or 5-carboxyfluorescein, succinimidyl ester (FITC) (Molecular Probes, OR) following methods of Harlow and Lane (1988).

Monovalent Fab fragments rather than whole IgGs were used in the perturbation experiments, because the two antigen binding sites of a whole IgG can crosslink membrane surface molecules, eliciting perturbation effects by grossly distorting membrane topology. The Fab fragments of these mAbs were prepared from the ascites fluid according to the description by Harlow & Lane (1988). Ascites fluid

was precipitated with 40% saturated ammonium sulfate with gentle shaking on ice for 24 hrs, followed by dialysis against PBS/4 mM EDTA at 4°C for 36 hrs. Then the dialyzed ascites was digested with mercuripapain (Worthington Biochemical Co., Freehold, NJ), followed by dialysis again against 5mM Tris-HCl buffer (pH8.0) overnight at 4°C. MAb ascites was then applied to a 2.5 ml DEAE cellulose (Whatman Bio. Ltd) column with gradient wash solution of 0 to 0.3M NaCl. The concentration of Fab fragments per fraction was estimated assuming that 1mg/ml of immunoglobulin has an absorption of 1.4 at 280 nm, measured by spectrophotometer (DU series 62, Beckman). As determined on silver-stained sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), Fabs have a molecular weight of 55 kDa (Harlow & Lane, 1988).

Immunocytochemistry

Antibody staining of fixed specimen: germinal plates were washed with 0.9% NaCl, 50 mM phosphate buffer (PBS, pH 7.4), and fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 30 min at room temperature. Embryos were post-fixed with absolute methanol for 15 min followed by extraction in absolute xylene for 5 min. The specimens were rehydrated with absolute methanol, 70% ethanol, and PBS. Antibodies and avidins were diluted with PBS/3% bovine serum albumin (Sigma) /2% Triton X-100 (Sigma). All except the final rinsing step during the antibody staining procedures were carried out with the same buffer. The final rinse was performed with PBS. For staining sensory afferents with a single mAb, germinal plates were

incubated overnight with hybridoma supernatant (1/20). After rinsing, embryos were incubated for 3 hr with biotinylated $(Fab')_2$ (1/100; Dako Corp., Carpenteria, CA). For double labeling sensory afferents with two different mAbs, germinal plates were first incubated overnight with the CE2 mAb (ascites fluid, 1/600). After rinsing, the specimen were incubated for 2 hr with FITC-conjugated rabbit anti-mouse IgG (1/50) (Dako Corp). After rinsing, the specimens were treated with ascites fluid of another mouse mAb (1/10%) to block anti-mouse IgGs. Next, the germinal plates were incubated for 4 hr in biotinylated CE1 mAb (1/200). After rinsing, they were treated for 1 hr with avidin-FITC (1/600) (Vector Lab., Burlingame, CA).

Live sensory afferents were stained by incubating freshly dissected germinal plates for 4 hr in L-15 to which fluorescently-conjugated mAbs were added. The CE1 subset was stained for its CE1 epitope with Laz2-369-RITC (1/20); the CE2 subset was stained for its CE2 epitope with Laz7-79-FITC (1/20).

The fluorescently-stained embryos were mounted with 70% glycerol to which saturating levels of p-phenylenediamine (Sigma; Cat. # P-1519) had been added to prevent fading of fluorescence.

Characterization of Carbohydrate marker CE2

Fluorescein-conjugated CE2 mAb was diluted (1/40) into L15 (no supplements) and different aliquots were preincubated for 12 hr with 20 μM of different neoglycoproteins (see below). Freshly dissected germinal plates were treated for 4 hr with just CE2 mAb

or with CE2 mAb that had been preincubated with different neoglycoproteins. The germinal plates were then rinsed, fixed in 4% paraformaldehyde, and embedded in 70 % glycerol to which p-phenylenediamine was added to prevent bleaching of fluorescence. The binding of mAbs to CE2 antigen was measured by assaying the intensity of sensory afferent staining in the different embryos under the confocal microscope.

Perturbation experiments

The perturbation experiments were performed on germinal plates that were conditioned to culturing by a 6 hr preincubation period, because no significant axonal growth occurs during the first 6 hr of culture (Song & Zipser, submitted, a). The sibling embryos of an 11.5 day old cocoon were divided into several groups that were cultured for an additional 6 hr in the absence of reagents (no treatment) or in the presence of Fab fragments (40 nM), neoglycoproteins (2 μM, Sigma), or enzymes (20 units/mL, Sigma). After rinsing, the embryos were fixed and sensory afferent projections were stained with either CE1 or CE2 mAb. The sensory afferent projections were analyzed under the Odyssey, a laser scanning confocal microscope (Noran, Madison, WI).

Data analysis of immunofluorescently stained tissue by the confocal microscope

The fluorescent stained projections of sensory afferents were analyzed under the Odyssey Real Time Laser Scanning confocal microscope (Noran instrument, Inc; Madison, WI) equipped with Image-1 Software (Universal Imaging Corp., PA), which allows us to capture serial images of sections to reconstruct the three-dimensional images and analyse the features of images. For fluorescein, the excitation and primary barrier filters were 488 and 515 nM, respectively. For rhodamine, the excitation and primary barrier filters were 529 and 550, respectively. A 100 x oil immersion objective (1.44 NA) was used for capturing images.

The ipsilateral neuropil of the embryonic CNS was optically sectioned in 1 μm increments. Axonal projections of sensory neurons were illustrated as reconstructed images that were formed by overlaying 12 serial optical sections. Optical sections of CNS devoid of fluorescently stained axons were captured ventral to the target region of sensory neurons and subtracted as background from the reconstructed images using the function "Graphic function: Logic Function" of Image-1. The background substracted reconstructed images were used to analyze the blocking of antibody binding to CE2 axons and the perturbation of CE2 axons with Fab fragments.

The blocking of antibody binding to CE2 axons with neoglycoproteins was quantified using the function "Brightness Measurement: Area Brightness" of Image-1. The intensity of fluorescently stained CE2 axons was measured in their neuropil

target region within a 10 x 20 μm area (medial-lateral x anterior-posterior) corresponding to the boxed area in Figure 1.

To quantify the perturbation results, we performed the function of Image-1 called "Brightness Measurement: Line Intensity Scan". To measure CE2 axons leaving their target region by crossing its medial border, we positioned a scanning line parallel to the medial border of the CE2 target region, but displaced by 5 μm. The scanning line was 232 pixels long (20 μm) and 1 pixel wide. The pixel value due to the fluorescent intensity of all the stained axons crossing the scanning line was measured. Furthermore, the average pixel value due to a single axon crossing the scanning line was measured. To estimate the total number of CE2 axons leaving their target region, the pixel value of the entire scanning line was divided by the average pixel value of a single fluorescent axon.

All the statistics were performed by Student T test (two tails).

Experiment reagents

The glycosidases and neoglycoproteins were purchased from Sigma, MO. β-N-Acetylglucosaminidase (Cat. # A2264); Cellulase (Cat. # C2415); β-Glucosidase (Cat. # G0395); α-Glucosidase (Cat. # G6136); β-Galactosidase (Cat. # G6512); α-Galactosidase (Cat. # G6762); α-Mannosidase (Cat. # M1266). Galactose-BSA (Albumin, Bovine-Galactosamide; 15-25 mol monosaccharide/mol albumin; Cat. # A5908); Fucose-BSA (Albumin, Bovine-Fucosylamide; 15-25 mol monosaccharide/mol albumin; Cat. # A6033), Cellobiosyl-BSA (Albumin, Bovine-Cellobiosyl; 15-25 mol disaccharide/mol albumin;

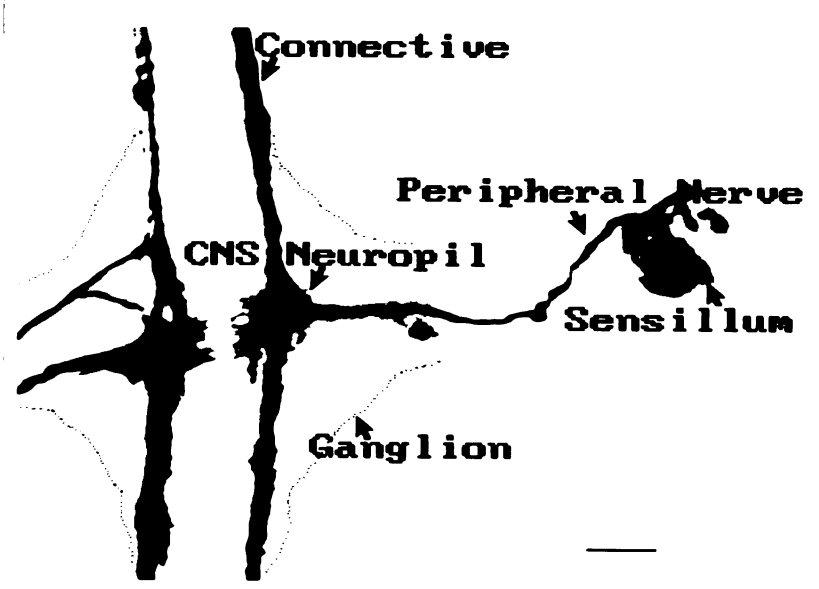
Cat. # A5408), Mannose-BSA (Albumin, Bovine-p-Aminophenyl-α-D-Mannopyranoside; 20-30 mol monosaccharide/mol BSA; Cat#. A4664), N-Acetyl-Glucosamine-BSA (Albumin, Bovine-p-Aminophenyl-N-Acetyl-β-D-Glucosaminide; 15-25 mol monosaccharide/mol albumin; Cat. # A1034), Glucose-BSA (Albumin, Bovine-Glucosamide; 15-30 mol monosaccharide/mol albumin; Cat. # A 6158).

RESULTS

During embryogenesis, the peripheral cell bodies of sensory afferents project their axons into target regions of the segmental ganglia of the leech CNS (Figure 13). Two days after sensory afferents project into the neuropil, they begin to express different carbohydrate epitopes. A large subset of neurons, the putative mechanodetectors, express CE1 (carbohydrate epitope 1), while a medium size subset, the putative chemodetectors, express CE2 (carbohydrate epitope 2). The subregions of the sensory neuropil occupied by these two different subsets was previously analyzed in adult segmental ganglia (Peinado, et al., 1987; Zipser, et al., 1994). The ventral sensory neuropil is dominantly colonized by the more numerous CE1 axons, while both subsets cohabitate in the other two regions of the sensory neuropil, however demonstrating different degrees of spatial autonomy. In the medial sensory neuropil, they occupy opposite poles showing only minor spatial overlap at their junction. In contrast, in the dorsal sensory neuropil, the CE1 and CE2 axons experience a significant degree of spatial overlap. Thus, depending on the particular sensory neuropil region, the CE1 axons either provide the dominant input or both CE1 and CE2 axons provide input through converging or diverging projections.

To determine the spatial and temporal relationship of CE1 and CE2 axons during early embryogenesis, we double-labeled leech embryos with monoclonal antibodies recognizing their carbohydrate markers. The large subset of CE1 axons was stained with FITC-

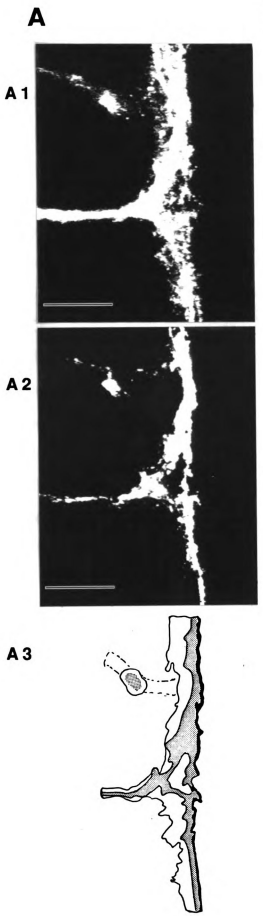
Figure 13. Properties of leech sensory afferent neurons. Leech sensory afferents that are associated with epithelial layers transduce different sensory modalities (Peinado, et al., 1987; Zipser, Erhardt, Song, Cole, & Zipser, 1994). On their cell surface, they express carbohydrate epitopes that are located on N-linked carbohydate chains of their 130 kD proteins. CE0, a mannose-containing epitope, is shared by the full set of these sensory neurons. In contrast, carbohydrate epitopes CE1, CE2, and CE3 delineate discrete subsets of these sensory neurons, the putative mechano-, chemo-, and heat detectors. As shown in this conforcal image stained by fullsett CE0 mAb Lan3-2, the peripheral cell bodies of sensory afferents project their axons into the synaptic neuropil of a segmental ganglion that is part of the leech CNS. Here we are studying the molecular mechanism of targeting of subset CE1 and CE2 axons that mediate both their convergent and divergent projections into different target regions during early embryogenesis. The stained specimen is from a 11 day embryo, midbody ganglion 15. Bar, 20 μm.


Connective
Peripheral Nerve
CNS Neuropil
Sensillum
Ganglion

labeled CE1 antibody while the smaller subset of CE2 axons was stained with RITC-labeled CE2 antibody. Using confocal microscopy, the sensory afferent projections were recorded in the left half of the neuropil of embryonic ganglia optically sectioned at 1 micron increments, and the images presented by overlaying 12 serial optical sections (Figure 14A1, A2). The overlap between the two subsets is diagramatically represented (Figure 14A3), demonstrating that much of the target area of the CE2 axons coincides with the lateral aspect of the CE1 target area. Thus, in 12 to 14 d old embryo, the lateralmost sensory neuropil is only occupied by CE1 axons and the most medial sensory neuropil is only occupied by CE2 axons while the two subsets overlap in between. The degree to which the smaller CE2 subset spatially overlaps with the larger CE1 subset was separately measured at each focal plane (n = 15 ganglia) and found to be 87%. Thus, in contrast to their more disparate distribution in the adult neuropil (Peinado, et al., 1987; Zipser, et al., 1994), during early embryogenesis, the smaller subset of CE2 is almost fully nested in the larger subset of CE1 axons.

During embryogenesis, the two different subsets appear along similar rostrocaudal gradients in the 32 segmentally reiterated ganglia of the leech CNS (Figure 14B). Between embryonic day 9 and 10, CE1 and CE2 axons are detected in the more anterior ganglia, the four ganglia that fuse to form the head ganglia as well as in the immediately following midbody ganglia. During the next three embryonic days, the two subsets complete their innervation of all midbody ganglia. Thus, the two subsets appear approximately at the same time in a given segmental ganglion suggesting that the

Figure 14. Developmental and molecular properties of two discrete subsets of sensory afferent neurons. The spatial overlap of the target regions of CE1 and CE2 axons during early embryogenesis was determined by double-labeling 12 day old embryos with mAbs. CE1 axons were labeled with FITC conjugated CE1 mAb, while CE2 axons were labeled with RITC conjugated CE2 mAb. The CNS neuropil of the double-labeled embryos was optically sectioned under the confocal microscope at 1 micron steps; the confocal images shown are the sum of 12 serial sections.
A. The large CE1 subset axons occupy a larger target region (A1) than the medium-size CE2 subset axons (A2). Bar, 10 μm. The spatial overlap of the FITC and RITC labeled axons is shown diagramatically with CE1 axons in light grey, CE2 axons in black, and their area of overlap in dark grey (A3). 82% of the target region occupied by CE2 axons overlaps with that of CE1 axons. **B**. The appearance of the CE1 and CE2 axons in the 32 segmental ganglia of the leech CNS were studied in 9 to 13 day embryos. Between embryonic day 9 and 10, the two neuronal subsets appear in the first 11 anterior ganglia which include head ganglia 1-4 and the first 7 midbody ganglia. Between day 10 and 13, CE1 and CE2 axons appear in the remainder of the midbody ganglia. After day 13, they appear in the 7 tail ganglia (not shown here). **C**. The carbohydrate contents of CE2 were determined by competing the binding of CE2 mAb to CE2 axons with different neoglycoproteins (20 μM) (n=7). Preincubating CE1 mAb with glucose-BSA (Glc-BSA) and with galactose-BSA (Gal-BSA) reduced the staining of CE2 axons by 60% as compared to control values. Preincubating CE2 antibody with two other neoglycoproteins, mannose-BSA (Man-BSA) and cellulose-BSA (Cell-BSA) did not significantly reduce the staining of CE2 axons.


A
A1
A2
A3
B
Innervated Ganglia
Midbody G1-21
Head G1-4
CE1 axons
CE2 axons
Age (Embryonic Day)
C
Staining Intensity (%)
*P<0.05
Control
Glc-BSA
Gal-BSA
Man-BSA
Cell-BSA

difference in their target assembly are not due to temporal but rather due to molecular interactions of their surface molecules.

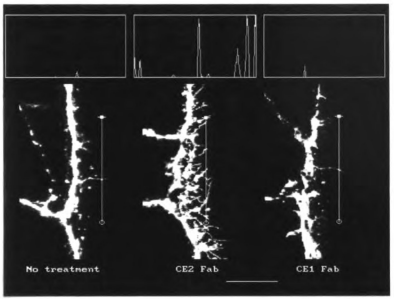
Previously, it had been shown that CE1, the surface marker of the putative mechanodetectors, contains α-extended galactose structures. Here we characterize the chemical composition of CE2 by competing the binding of its respective mAb (CE2 mAb) with different carbohydrates (Figure 14C). Embryos were briefly treated with only CE2 antibody or with CE2 antibody that had been preincubated with neoglycoproteins which are monosaccharides linked multivalently to a carrier protein such as BSA. The staining of CE2 axons was significantly reduced by the presence of galactose-BSA (20 μM) and glucose-BSA (20 μM) by 65 and 63%, respectively, but not the presence of cellobiosyl-BSA (20 μM). This suggest that both galactose-BSA and glucose-BSA can bind to the CE2 mAb, thereby preventing it from binding to CE2 on the surface of axons. This indicates that CE2 contains both galactose and glucose, in contrast to CE1, which apparantly contains only galactose (Song & Zipser, submitted).

To investigate whether CE1 and CE2, the two neuronal subset markers, are responsible for directing the targeting of their respective subsets, we separately blocked these epitopes with their respective Fab fragments while culturing the germinal plate of the embryonic leech for 6 hr. When cultured in the enriched growth medium alone, CE2 axons normally assemble in their target region (Figure 15A, left panel) in the synaptic neuropil. However, the addition of CE2 Fab fragments (40 nM) led to a massive scattering of axons across the entire ipsilateral neuropil (middle panel; CE2 Fab).

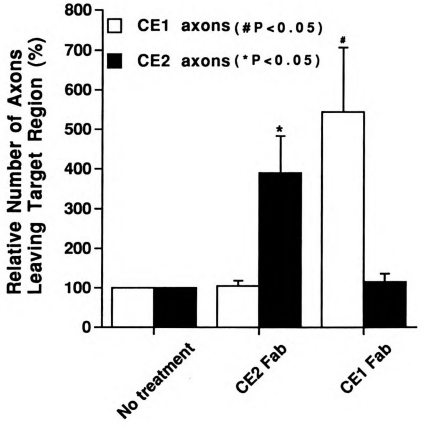
Figure 15. The targeting of neuronal subsets is mediated by their respective developmentally regulated carbohydrate markers. Sibling embryos were divided into three groups, a control group that was cultured in the enriched medium alone and two experimental groups that were cultured either in the presence of CE2 Fab or CE1 Fab fragments (40 nM) (n=8). Afterwards, the perturbation effects were assayed in optical sections taken with a confocal microscope. **A**. CE2 axons normally assemble in their target regions in embryos grown in the enriched medium only (no treatment), or in the presence of CE1 Fab fragments. In contrast, CE2 axons leave their target regions and disperse across the neuropil in embryos grown in the presence of CE2 Fab fragments. The number of axons leaving their target region was quantified by performing a line scan along a scanning line that is positioned in parallel to the CE2 target regions but displaced medially by 5 μm. The panels above the neuropil images illustrate the pixel intensity value due to axons crossing the scanning line. The two small peaks in the middle panel and the single peaks in the outside panels represent pixel intensity values of single axons. The larger peaks in the middle panel represent small bundles of axons. To determine the number of axons leaving their target regions during perturbation, the whole pixel intensity value of the entire scanning line was divided by the average pixel intensity value of the single axons that was obtained by averaging ten single axons. In the experiment illustrated here, an estimated 49 axons cross the scanning line in the presence of CE2 Fab fragments. Bar, 10 μm. **B**. Sibling embryos were cultured in the enriched medium alone or treated with CE1 and CE2 Fab fragments. The number of axons leaving their target region in embryos cultured in the absence of Fab fragment was set to 100%. There was a 544 % ± 162% (n=8) increase in CE1 axons leaving their target regions in the presence of CE1 Fab, and a 390% ± 93% (n=8) increase in CE2 axons leaving their target regions in the presence of CE2 Fab fragments. CE1 axons were not perturbed by CE2 Fab fragments, and CE2 axons were not perturbed by CE1 Fab fragments.

CE2 axons

A


No treatment
CE2 Fab
CE1 Fab

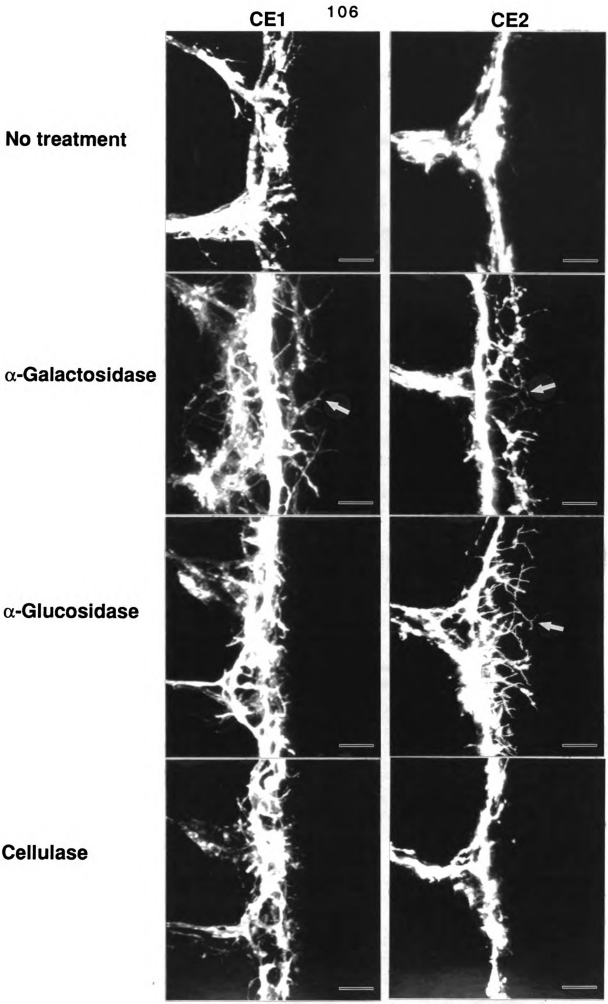
B


CE1 axons (#P<0.05)
CE2 axons (*P<0.05)
Relative Number of Axons Leaving Target Region (%)
800
700
600
500
400
300
200
100
0
*
#
No treatment
CE2 Fab
CE1 Fab

In contrast, the targeting of these CE2 axons was not perturbed by treating embryos with Fab fragments that are specific for a different neuronal subset, the subset of CE1 axons (right panel; CE1 Fab). The number of CE2 axons leaving their target region under the different experimental and control conditions was counted by performing a line scan along a scanning line positioned parallel to the CE1 target region, but medially displaced by 5 m. The application of CE2 Fab fragments led to a four-fold increase in axons leaving the CE2 target region and crossing the scanning line (Figure 15B) as compared to the controls. Thus, The targeting of CE2 axons is specifically mediated by the unique carbohydrate on their cell surface. Likewise, CE1 axons were specifically perturbed by CE1 Fab but not by CE2 Fab. Thus, the targeting of the two different axonal subsets can be manipulated separately by blocking the interaction of their specific subset markers. This suggests that each carbohydrate marker independently controls the targeting of the neuronal subset by which it is expressed.

We characterized the chemical nature of the targeting response of the two different neuronal subsets with perturbation assays using glycosidases and neoglycoproteins. Endogeneus carbohydrate structures were modified by culturing germinal plates in the presence of seven different glycosidases, followed by the staining of either CE1 or CE2 axons. As illustrated in the confocal images (Figure 16), cellulase did not perturb the targeting of either CE1 or CE2 axons. The targeting of CE1 axons was specifically affected by the presence of α-galactosidase, but α-glucosidase had no significant effects. In contrast, the targeting of CE2 axons was

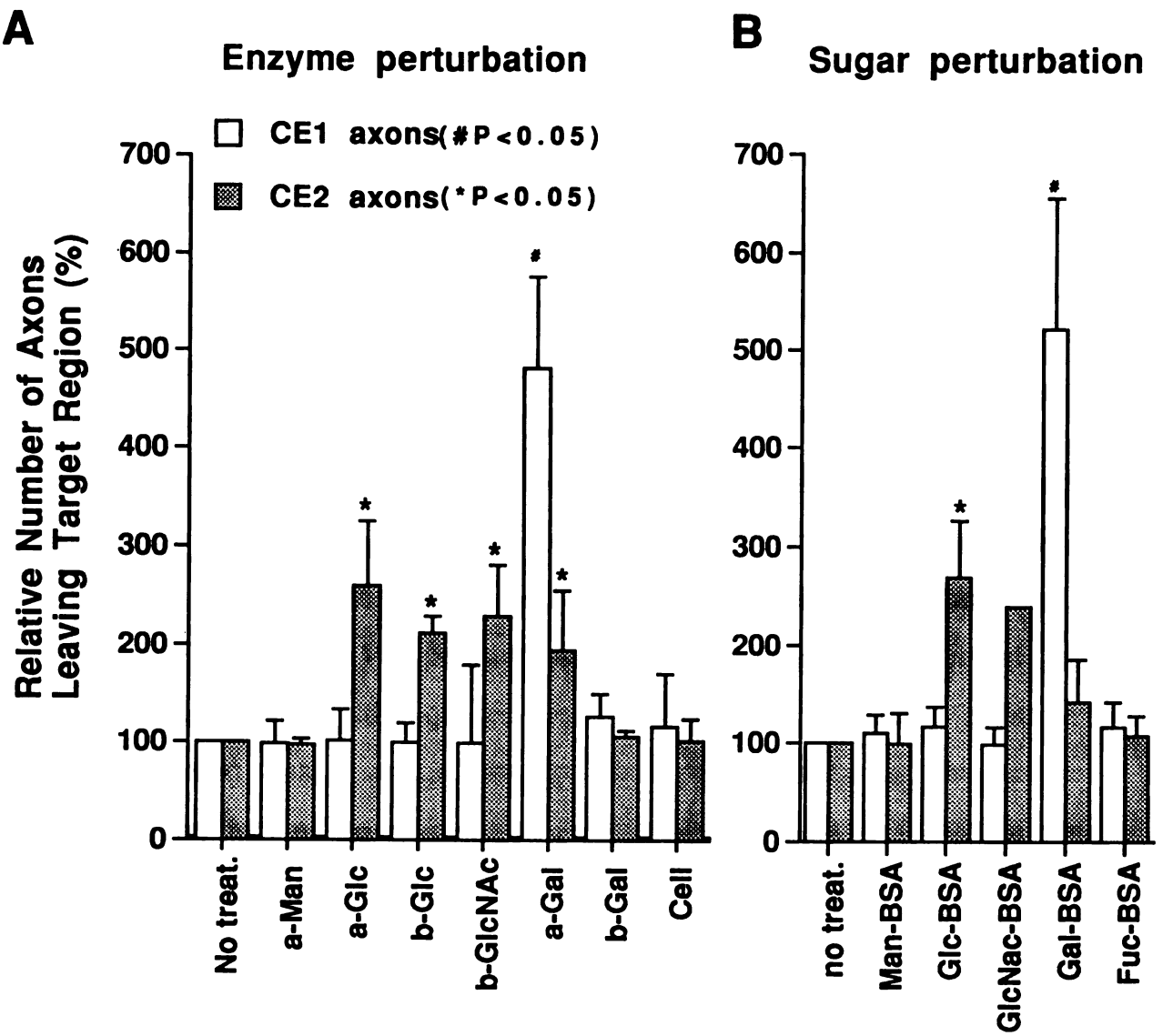
Figure 16. Perturbation of axonal subsets with glycosidases. Sibling embryos were divided into four groups that were cultured in the absence of glycosidases or in the presence of 20 units/ml of either α-galactosidase, α–glucosidase or cellulase. After fixation, the embryos were either stained for CE1 or CE2 axons. In the absence of glycosidases, CE1 and CE2 axons assemble normally in their target regions (the top row). The presence of α–galactosidase led to both CE1 and CE2 axons leaving their target regions (the second row, arrows). The presence of α–glucosidase only led to CE2 axons leaving their target region (arrow) but did not affect the targeting of CE1 axons (the third row). The presence of cellulase did not affect either subset (the bottom row).


CE1
CE2
No treatment
α-Galactosidase
α-Glucosidase
Cellulase

sensitive to both α-galactosidase and α-glucosidase. The effects elicited by the different glycosidases on CE1 and CE2 axons were quantified (Figure 17A) by performing lines scans parallel to their respective target regions as used during the Fab fragment perturbation experiment. Exposing cultured embryos to either α- or β-glucosidase or β-N-acetylglucosidase, led to a two to three fold increase of CE2 axons leaving their target region. Likewise, treatment with α-galactosidase also led to a three fold increase in axons leaving their target region. However, the targeting of CE2 axons was not perturbed by β-galactosidase, mannosidase or cellulase.

Neoglycoproteins were applied to the cultured embryos to compete with endogeneous carbohydrate structures for carbohydrate-binding proteins (Figure 17B). These sugar perturbation experiments confirmed that the targeting of CE1 axons is mediated by galactose-specific recognition mediates Only galactose-BSA but not the four other neoglycoproteins affected the targeting of CE1 axons. Likewise, treating embryos with glucose or N acetylglucosamine confirmed that the targeting of CE2 axons involves a glucose-recognition. However, the targeting of CE2 axons was not significantly perturbed by galactose-BSA. Thus, the targeting of CE2 axons is mediated primarily by glucose-recognition with only a minor galactose-component.

Figure 17. Quantification of the perturbation of CE2 axonal targeting by neoglycoproteins and exoglycosidases.
A. For the enzyme perturbations (n =7), sibling embryos were cultured in the enriched medium in the presence of 20 units/ml of the following exoglycosidases: α–mannosidase, α–glucosidase, β–glucosidase, β-N-acetylglucosaminidase, α–galactosidase or β–galactosidase.
B. For the neoglycoprotein perturbations (n=7), sibling embryos were cultured in the presence of 2 μM of the following neoglycoproteins: mannose-BSA, glucose-BSA, N-acetylglucose, galactose-BSA or fucose-BSA. Each experiment included a no treatment group of 3 sibling embryos cultured in the enriched medium only. The sibling embryos were stained for either CE1 or CE2 axons. The number of axons leaving their target regions in the control embryos (no treatment) or in the experimental embryos was estimated by performing a line scan on optically sectioned embryonic CNS. CE1 axons (grey) leave their target regions only when embryos are treated with α–galactosidase (by 481% ± 95.5%, n=7) or galactose-BSA (by 521% ± 134%, n=7), indicating that the targeting of CE1 axons is mediated by galactose-specific recognition. In contrast, CE2 axons (black) leave their target region when exposed to different glucosidases (n=7) such as α–glucosidase (by 260% ± 65%), β–glucosidase (by 212% ± 17%), β-N-acetylglucosaminidase (by 229% ± 52%), and α–galactosidase (by 194% ± 61%), suggesting that both galactose and glucose components are contained in carbohydrate marker CE2. However, only competing for the carbohydrate-binding proteins with glucose-BSA and N-acetylglucosamine-BSA, but not with galactose-BSA, perturbs the targeting of CE2 axons suggesting that the glucose-specific recognition is of higher significance in their targeting than the galactose-specific recognition.


A
Enzyme perturbation
CE1 axons(#P<0.05)
CE2 axons(*P<0.05)
Relative Number of Axons Leaving Target Region (%)
700
600
500
400
300
200
100
0
No treat.
a-Man
a-Glc
b-Glc
b-GlcNAc
a-Gal
b-Gal
Cell
B
Sugar perturbation
700
600
500
400
300
200
100
0
no treat.
Man-BSA
Glc-BSA
GlcNac-BSA
Gal-BSA
Fuc-BSA

DISCUSSION

During the development of the leech nervous system, carbohydrate epitopes that mark sets and subsets of sensory neurons play critical roles in helping these neurons to project into their proper target regions. Previously, we reported that the targeting response of sensory neurons consist of two sequential steps that are mediated by their function-specific carbohydrate markers CE0 and CE1 (Zipser & Cole, 1991; Song & Zipser, 1993). The initial projections of generic sensory neurons into the neuropil is mediated by CE0, their constitutive mannose-containing marker. The subsequent assembly of a large subset of these neurons into a restricted target region is mediated by CE1, the subset's developmentally regulated, galactose-containing marker. Here we characterized the medium size subset of sensory neurons, marked by CE2, to compare its cellular and molecular properties to those of the large CE1 subset. We present evidence that the two subsets of sensory neurons, although sharing a number of properties, differ in a feature that has a critical impact on their targeting responses.

The two neuronal subsets are detected at about the same time in a given segmental ganglion through the expression of CE1 and CE2, their definitive subset carbohydrate markers. Preliminary evidence suggests that a third subset identified by CE3 is also detectable at the same time. Previous work showed that the innervation of a ganglion by generic sensory neurons, identified via their mannose-specific epitopes, precedes the appearance of the CE1 subset by about two days (Song & Zipser, submitted, c). The question arises

whether subset neurons differentiate two days after the generic sensory neurons project into the neuropil or whether generic sensory neurons express one of the three subset carbohydrate markers, either CE1, CE2 or CE3, two days after they arrive in the neuropil. Our previous evidence that the number of neurons expressing the mannose epitope is approximately the same as the number of neurons that express the three subset carbohydrate markers (Zipser, et al., 1994) is consistent with a developmental regulation of expression of subset markers on generic sensory neurons. The chemical composition of these developmentally regulated subset markers, containing α-galactose versus α-galactose and glucose, suggest that they are of the hybrid or complex type in contrast to the full set marker that is of the high mannose type.

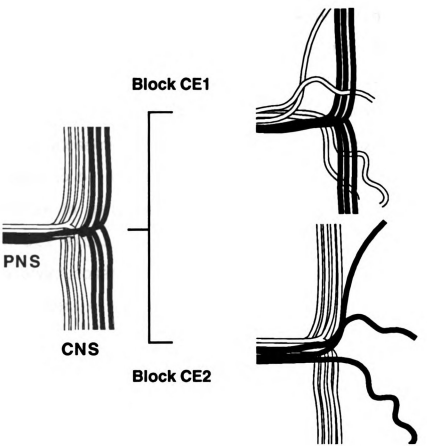
Both neuronal subsets densely group into stereotypic target regions via carbohydrate interactions mediated by their respective subset epitopes. Blocking the carbohydrate epitope of a given subset with its respective Fab fragment only perturbs the targeting of axons belonging to the same subset, sparing the axons of the other subsets (Figure 18). The specificity with which each carbohydrate marker mediates the targeting of its axonal subset was determined by delicate perturbations using only nanomolar amounts of Fab fragments.

However, the two subsets differ in the complexity of their carbohydrate-mediated interactions as demonstrated by perturbing their targeting with neoglycoproteins and glycosidases. The targeting mechanism of the CE1 subset appears to be simpler because it involves only one type of carbohydrate recognition. CE1 axons are

Figure 18. Schematic representation of the perturbation experiments and the underlying molecular interactions mediating the grouping of CE1 and CE2 axons into their respective target regions within the CNS synaptic neuropil. Blocking CE1 with CE1 Fab fragments leads to the dispersal of only CE1 axons (white), and blocking CE2 with CE2 Fab fragments leads to the dispersal of only CE2 axons (black).

Targeting of CE1 axons: galactose-specific recognition

Targeting of CE2 axons: mixed, glucose/galactose-recognition



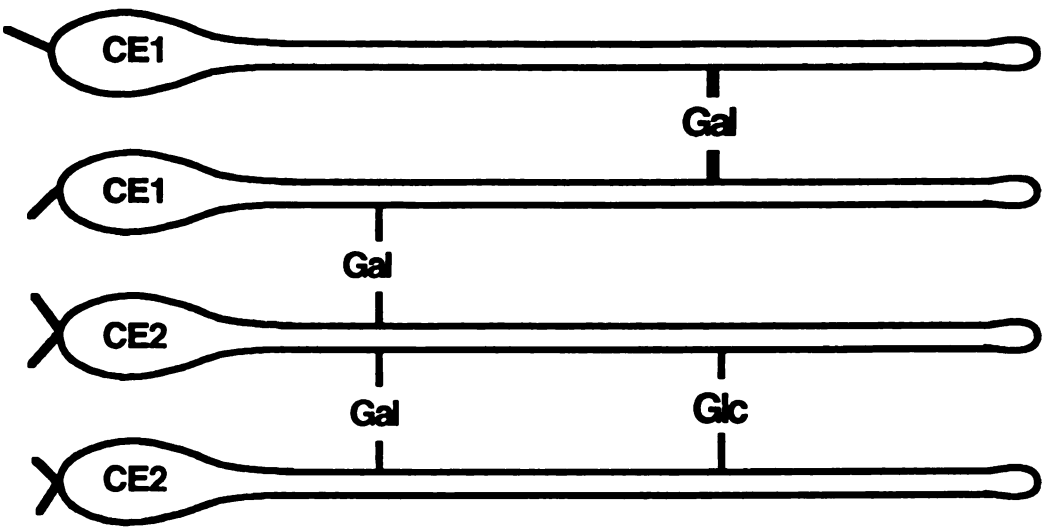
only perturbed by the application of a galactosidase and galactose-BSA but not by other enzymes and sugars. The finding that the magnitude of the galactosidase and galactose-BSA perturbations parallels that of the Fab fragment perturbation is consistent with CE1 targeting relying solely dependent on galactose-recognition (Song & Zipser, submitted, c).

In contrast, the targeting mechanism of the CE2 axons appears to be more complex because it involves several types of carbohydrates whose interactions may be of different significance. The critical involvement of glucose-specific recognition in the targeting of CE2 axons is suggested by their sensitivity to α–, β–glucosidases, β–N-acetylglucosaminidase, glucose-BSA and N-acetylglucosamine-BSA. In contrast, galactose-specific recognition appears to be of lesser significance because CE2 axons are only sensitive to galactosidase but not to galactose-BSA. The finding that CE2 axons are less strongly perturbed by galactose, glucose or N-acetylglucosamine than by Fab fragments is consistent with the involvement of more than one type of carbohydrate-mediated interaction in the targeting of CE2 axons (Figure 19). It remains to be seen whether the different sugar perturbations are additive and can reach the degree of perturbation elicited with Fab fragments.

It is open to speculation whether the carbohydrate recognition events mediating the targeting of the two neuronal subsets involve heterotypic or homotypic interactions. Originally, carbohydrate recognition was considered to be the heterotypic interaction of a carbohydrate with a carbohydrate-binding protein or lectin. Classical examples are the bacterial infection of the gut (Ofek &

Figure 19. The binding interactions among CE1 and CE2 axons that are diagramatically illustrated here are consistent with data from the neoglycoprotein and enzyme perturbation experiments. CE1 axons interact with one another via galactose-specific recognition. CE2 axons interact with one another via both galactose- and glucose-specific recognition. CE1 and CE2 axons interact with one another via galactose-specific recognition.

Carbohydrate recognitions among CE1 and CE2 axons



Sharon, 1988; Sharon, 1987), hepatic clearance of serum asialoglycoproteins (Ashwell & Harford, 1982; Ashwell & Morell, 1974), embryo compaction and sperm fertilization (Dutt, Tang, & Carson, 1987; Bayna, Shaper, & Shur, 1988). In the nervous system, L1 and NCAM were found to interact homotypically via a heterotypic mannose-specific recognition (Horstkorte, et al., 1993). A more recent idea is that carbohydrate recognition can also be based on homotypic interactions. The original cell type on which cell adhesion was studied, namely marine sponges, have now been shown to interact homotypically via the same glycan expressed on all sponge cells (Misevic & Burger, 1993). Another example is the homotypic interaction of CD15, also called SSEA-1, that plays a role in embryo compaction or the aggregation of F9 teratocarcinoma (Hakamori, 1992). While the homotypic interactions are of very high specificity, they are considered to be of low affinity, depending on multivalency of binding. In contrast, carbohydrate-lectin binding is considered to be of higher affinity but lower specificity. Perhaps one could consider the polysialic acid-mediated modulation of NCAM binding, which plays an important role in the fasciculation and branching of axonal bundles of motorneurons (Landmesser, Dahm, Schultz, & Rutishauser, 1988; Landmesser, et al., 1990) to be a homotypic interaction.

In the leech nervous system, a homotypic interaction among the galactose-containing CE1 epitope on opposing axonal surfaces may mediate the fasciculation of CE1 axons in peripheral nerves or the grouping of axons in their target regions in the sensory neuropil. In contrast, a classical carbohydrate-lectin interaction may mediate

the final stabilization of axons in their target regions. A candidate lectin involved in axonal targeting is leech galectin (Cole & Zipser, 1994a; Cole & Zipser, 1994b). Likewise, the fasciculation of CE2 axons in peripheral nerves and the grouping of CE2 axons into their target region could be mediated by highly homotypic interactions of the CE2 epitope on opposing axonal surfaces. A potential receptor for heterotypic interaction, specifically a glucose-binding protein, remains to be identified.

The mixed carbohydrate interactions of the CE2 axons involving both the glucose and galactose on their surface could explain the observation that the smaller subset of CE2 axons is spatially overlapping with the larger subset of CE1 axons. CE1 and CE2 axons may be able to share similar neuropil territories because both are capable of galactose-specific interactions. It appears reasonable that CE2 axons overlap with CE1 axons in a distinct subregion of the CE1 territory rather than being spread diffusely through the entire CE1 territory because CE1 inherently interacts more strongly with CE1 while CE2 inherently interacts more strongly with CE2. Thus, mixed carbohydrate recognition mechanisms may provide an explanation for mechanisms that lead to the structuralization of synaptic neuropils. They may facilitate the innervation of a target region receiving multiple presynaptic inputs.

SUMMARY AND CONCLUSIONS

To study the roles of carbohydrate epitopes expressed on the neuron surface of leech sensory afferents in an intact nervous system, we devised an embryo culture system. Leech embryos cultured in the enriched medium grow nearly normal as at the physiologic condition in vivo. Using this tissue culture system, we studied the kinetics of molecular perturbation of Fab fragments on the sensory afferent projection in CNS neuropil. We further studied the roles of carbohydrate epitopes on the afferent surface in formation of neuronal connections in the CNS neuropil.

In leech sensory nervous systems, functional sets and subsets of adult leech sensory neurons are specifically marked with different glycoconjugates at the neuron surface (Table 1) (Peinado, et al., 1987; Peinado, Zipser, & Macagno, 1990; Bajt, Schmitz, Schachner, & Zipser, 1990b; Bajt, Cole, & Zipser, 1990a). Our experiments proved that these carbohydrate markers play important functions during the formation of neuronal connections in the CNS. A mannose-containing generic marker CE0 is expressed during the differentiation of sensory afferents and guides afferent projection while axons innervate the CNS neuropil (Zipser, et al., 1989; Zipser & Cole, 1991). Axons defasciculate and disperse across the CNS neuropil via a mannose-specific carbohydrate interaction involving CE0, that is the first step of process for the formation of neuronal networks. For the sensory information finally being channeled into specific target regions in the central nervous system, which is the case in leech

sensory nervous systems (Zipser, et al., 1994; Peinado, et al., 1987), the projection of axons in the CNS is further structuralized into multiple subregions; we call this the second step of the process of formation of neuronal networks in the leech CNS. During the shift of axonal projection patterns from the first step, defasciculation, to the second step, assembling to target regions, subset afferents with different modalities expressed specific subset carbohydrate markers. A large subset of afferents with mechanical detector express carbohydrate marker CE1, while a medium subset of afferents with chemo-detectors express carbohydrate marker CE2.

We characterized these two carbohydrate marker and determined CE1 as being galactose-specific and CE2 as being glucose/galactose-specific. Our molecular perturbation experiments demonstrated that subset carbohydrate markers CE1 and CE2 are specifically involved in the structuralization of target subregions, and respectively, guide CE1 and CE2 subset axons innervating their own specific targeting regions (Song & Zipser, submitted, b.; Song & Zipser, submitted, c). So, during leech embryogenis, early expressed fullset carbohydrate makers CE0 collaborate with later expressed subset carbohydrate markers CE1 and CE2 to precisely form the CNS sensory neural connection.

This work, to our knowledge, is the first direct demonstration of a molecular signal of glycoprotein physiologically regulating the neuronal projection. The monoclonal antibodies against fullset and subset sensory afferents (Zipser & McKay, 1981) and the fine-tuned leech embryo culturing system (Song & Zipser, submitted, a) made this study possible.

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