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The restricted spatial and temporal expression of a nervous-system-specific antigen involved in axon outgrowth during development of the grasshopper

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Summary

To identify molecules important for pathfinding by growing axons, monoclonal antibodies (mAb) have been generated against embryonic grasshopper tissue. One mAb, 2B2, shows labeling exclusively in the nervous system. It recognizes a surface epitope on neuronal growth cones, filopodia and axons in the central nervous system (CNS). Initially, the antigen is expressed on all processes of the CNS; after 70% of embryonic development, localization of the 2B2 mAb is restricted to a small subset of axon tracts within the ganglia. Immunoprecipitation from embryonic membrane extracts with the 2B2 mAb reveals a unique band of $160\times10^3 M_r$. Functional studies with the 2B2 mAb demonstrate that the antigen is important in growth cone–axon interactions during process outgrowth. Growth cones that extend along axonal substrata are either blocked in growth or grow along an aberrant pathway when embryos are cultured in the presence of the 2B2 mAb. However, pioneer neurons that extend processes on non-neuronal substrata grow normally.

Key words: axon subset, grasshopper, axonogenesis, pathfinding, monoclonal antibody, axon outgrowth, insect.

Introduction

The stereotyped complexity of the central nervous system demands precise instructions to ensure that the correct neuronal connections are formed during development. There are at least two different types of environments present in the embryonic grasshopper through which neurons must navigate; each presents different pathfinding problems. The first neurons to extend processes are presented with an environment devoid of other axons. These pioneer neurons must navigate across substrata consisting of nerve cell bodies, glial cells, epithelial cells and basal lamina. Neurons that extend their processes at a later time in development navigate in an environment rich in axons. Many of these later arising neurons send their processes along and fasciculate with an already established neuronal scaffold of axon fascicles (Goodman *et al.* 1982). A single process may grow sequentially along many different fascicles, leaving one axon substratum for another at particular points in order to reach its target eventually. It is the specific sequence of choices of axon substrata that lead most neurons to their correct target.

Since the use of monoclonal antibodies was introduced into the field of neuroscience (Barnstable, 1980; Zipser and McKay, 1981), numerous molecules that show regional expression in the nervous system have been identified. Of these, several are cell surface proteins with a proposed function in cell recognition or cell adhesion. These include molecules in vertebrates such as N-cadherin (Takeichi, 1988), N-CAM (Edelman, 1988), L1/NILE/69A1 (Rathjen and Schacher, 1984; Fischer *et al.* 1986; Stallcup and Beasley, 1985), G4/Ng-CAM/8D9 (Rathjen *et al.* 1987*a*; Chang *et al.* 1987; Hoffman *et al.* 1986; Lagenaur and Lemmon, 1987), F11 (Rathjen *et al.* 1987*a*), neurofascin (Rathjen *et al.* 1987*b*), contactin (Ranscht, 1988; Ranscht *et al.* 1984), F3 (Gennarini *et al.* 1989*a*; Gennarini *et al.* 1989*b*), TAG-1 (Dodd *et al.* 1988), RB-8 (Schwob and Gottlieb, 1986, 1988), and axonin-1 (Ruegg *et al.* 1989); molecules isolated from invertebrates include amalgam (Seeger *et al.* 1988), neuroglian (Bieber *et al.* 1989), fasciclin III (Patel *et al.* 1987) in *Drosophila*; fasciclin I and II in grasshopper (Bastiani *et al.* 1987; Zinn *et al.* 1988; Harrelson *et al.* 1988), $130\times10^3 M_r$ glycoproteins in leech (Peinado *et al.* 1987; Hogg *et al.* 1983), and the 5B12 antigen in cricket (Meyer *et al.* 1988). Many of these molecules are also present in tissues outside of the nervous system. Of the molecules for which the distribution in many tissues has been examined in vertebrates, TAG-1 (Dodd *et al.* 1988), contactin (Ranscht *et al.* 1984) and RB8 (Schwob and Gottlieb, 1986) have been reported to be restricted to nervous system tissue. In invertebrates, no cell surface proteins

present on neurons in the CNS during axon outgrowth are reported to be nervous system specific. A molecule found only in the nervous system may have evolved to serve a function unique to its development as a tissue. Here we report an antigen that is expressed only in the nervous system during embryogenesis of the grasshopper.

The relatively simple and highly accessible nervous system of the embryonic grasshopper and the detailed information about cell body location, axonal projection and developmental time course of outgrowth of many identified neurons provides a strong model system in which to study neuronal pathfinding. To identify molecules present in the embryonic environment of the grasshopper that are involved in the process of neuronal pathfinding, we generated monoclonal antibodies (mAbs) against grasshopper embryonic nerve cord. In this paper, we describe the antigen recognized by the 2B2 mAb whose pattern of expression changes during embryogenesis and whose function may be important in the development of the central nervous system.

Materials and methods

Generation of mAb

50 nerve cords from the grasshopper (*Schistocerca americana*) at 50% of embryonic development were dissected in Ringers solution (8.76 mg ml^{-1} NaCl, 0.22 mg ml^{-1} KCl, 1.15 mg ml^{-1} TES, 0.29 mg ml^{-1} $CaCl_2.2H_2O$, 0.25 mg ml^{-1} $MgSO_4.7H_2O$, pH 7.3–7.5), homogenized in phosphate-buffered saline (PBS) (2.13 mM NaH_2PO_4, 15.8 mM Na_2HPO_4, 175 mM NaCl, pH 7.4) with 5 mM EDTA, 20 μg ml^{-1} phenylmethylsulfonyl fluoride, and a cocktail of protease inhibitors (1 μg ml^{-1} of antipain, chymostatin, leupeptin, pepstatin, *N-p*-tosyl-L-lysine chloromethyl ketone, and *N*-tosyl-L-phenylalanine chloromethyl ketone). The homogenate was then centrifuged at 1000 *g* (4°C) for 10 min. The supernatant was centrifuged at 200 000 *g* (4°C) for 1 h. The membrane pellet was resuspended by sonication in 100 μl of PBS and an equal volume of Freund's adjuvant, and injected intraperitoneally into a BALB/c mouse (approximately 1 mg of protein per injection). The first injection was with Freund's complete adjuvant; the remainder were with Freund's incomplete adjuvant, except for the final injection without adjuvant 3 days before the fusion. The mouse was injected at 3-week or longer intervals, and received 5 injections before fusing its spleen cells with NS-1 myeloma cells (Kohler and Milstein, 1975; Oi and Herzenberg, 1980).

Screening of mAbs

Hybridoma supernatants were screened histochemically on whole-mount 40% grasshopper embryos. Embryos were fixed in 2% paraformaldehyde in Millonig's buffer for 15–30 min, washed in PBS with 1 mg ml^{-1} glycine for 10 min, washed in PBS with 2% bovine serum albumin and 0.4% saponin (PBS+BSA+S) for 10 min, and incubated with equal volumes of the hybridoma supernatant and PBS+BSA+S overnight at 4°C. Embryos were then washed in PBS for 2 h, and were finally incubated in a blocking solution of PBS+BSA+S and 5% normal goat serum for another 15 min before being incubated for 2 h at 22°C (or overnight at 4°C) in a 1:100 dilution of FITC-labeled goat anti-mouse antibody (Jackson ImmunoResearch) in PBS+BSA+S. Embryos were washed in PBS for at least 2 h and then viewed with a 25× objective on a Leitz compound microscope.

Immunofluorescence and HRP immunocytochemistry for light microscopy

Grasshopper embryos were staged by comparison with a timetable prepared by Bentley *et al.* (1979). Whole embryos or dissected nerve cords were fixed in 2% paraformaldehyde in Millonig's buffer for 30–60 min, washed in PBS+1 mg ml^{-1} glycine for (10 min–1 h), blocked in PBS+20% normal goat serum+0.3% Triton X-100 for 15 min, and incubated in a 1:500 dilution of primary antibody overnight at 4°C. The embryonic tissue was then washed in PBS+0.3% Triton X-100 for 1 h followed by a second treatment in blocking solution.

Embryos prepared for immunofluorescence were incubated overnight at 4°C with a 1:200 dilution of goat anti-mouse secondary antibody conjugated with either fluorescein or rhodamine (Jackson ImmunoResearch). The embryos were washed as above and then mounted in 5% *n*-propyl gallate in 75% glycerol and viewed with a Leitz epifluorescence compound microscope.

Embryos (or nerve cords) prepared for HRP immunocytochemistry were labeled with primary antibody and then incubated overnight at 4°C with a 1:200 dilution of an HRP-conjugated goat anti-mouse antibody (Jackson ImmunoResearch). After washing, HRP was visualized using diaminobenzidine (DAB) (0.5 mg ml^{-1}) and hydrogen peroxide (0.003%). The reaction was stopped in PBS and embryos were cleared in 100% glycerol and viewed with Nomarski optics.

Immunoelectron microscopy

Live embryos were cultured in a RPMI 1640 (Cellgro)-based medium (Raper *et al.* 1984*b*) which was used in all incubation and washing steps. Incubation of the embryos in a 1:100 dilution of the 2B2 mAb for 2 h at room temperature was followed by several washes in media for 20 min, incubation in a 1:50 dilution of HRP-conjugated goat anti-mouse IgG for 2 h, and then washed again as above. Embryos were fixed in 1% paraformaldehyde and 1% glutaraldehyde in Millonig's buffer for 30 min. After washing with several changes of Tris-buffered saline (TBS) (50 mM Tris pH 7.2, 350 mM NaCl), 0.2% cobalt was added to the embryos for 10 min. The HRP was visualized using 1 mg ml^{-1} DAB and 1 unit ml^{-1} glucose oxidase in TBS (Raper *et al.* 1984*a*; Watson and Burrows, 1981). The embryos were allowed to react for 45 min, washed in TBS, fixed again in 1% paraformaldehyde and 1% glutaraldehyde for 1 h and washed in TBS. Embryos were incubated in 1% OsO_4 in TBS for 1 h, washed in TBS, stained in 2% uranyl acetate in TBS, washed in TBS and then dehydrated in an ethanol series. The embryos were embedded in an Epon–Araldite mix of plastic. Ultrathin sections were mounted on Formvar-coated slot grids and viewed on a Phillips 201 electron microscope.

Immunoprecipitation

A membrane preparation from 40% embryos was prepared by dissecting embryos into cold RPMI and 6 mg ml^{-1} glycine. Embryos were washed in 10 mM triethanolamine (TEA), 5 mM EDTA, and protease inhibitors (see above). The embryos were homogenized, spun at 1000 *g* to remove nuclei and organelles, and the supernatant was spun at 200 000 *g* to pellet the membrane fraction. The pellet was resuspended in 10 mM TEA plus protease inhibitors and labeled with ^{125}I using lactoperoxidase (Haustein *et al.* 1975), solubilized in IPB (10 mM TEA, 150 mM NaCl, 1% NP-40, pH 7.8), and then

subjected to precipitation using antibody preformed complexes as described by van Agthoven *et al.* (1981). The labeled membrane proteins were exposed sequentially to the 3B11 mAb, the 2B2 mAb, and then the 13E10 mAb. Before incubation with each antibody, the membrane preparation was cleared of antibody by incubation with Pansorbin cells, which have protein A expressed on the cell surfaces. The precipitated complexes were resuspended in 0.5% 2-deoxycholic acid (Sigma) in 10 mm TEA and then spun through a two-step sucrose gradient (10% sucrose in IPB over 20% sucrose in 10 mM TEA). The pellet was washed with 0.2% NP-40, 10 mM TEA and resuspended in SDS sample buffer. The precipitated proteins were analyzed by SDS–PAGE under reducing conditions on a 7.5% polyacrylamide gel and visualized by exposure to Kodak X-OMAT X-Ray film with an intensifying screen.

Antibody blocking experiments

35% embryos were removed from the egg case and yolk and placed in culture media (Chen and Levi-Montalcini, 1969) containing 50% Schneider's *Drosophila* medium (Gibco), 40% MEM (Gibco), 10% heat-treated fetal calf serum (Hyclone), 1.5 mg ml^{-1} bovine insulin (Sigma), 0.03 mg ml^{-1} glycine (Sigma), 0.005 μg ml^{-1} juvenile hormone (Sigma), 0.003 μg ml^{-1} β-ecdysterone (Sigma), and 10000 i.u. ml^{-1} antimycotic/antibiotic (Sigma) for 24–36 h at 30°C. Embryos were chosen from a single clutch of eggs, which are normally of a single developmental stage. To ensure that the embryos used for the experiment were within 1% of embryonic development, distances of axon outgrowth of the MP1 cell and the aCC cell were compared among several random embryos from a single clutch. This was done by injecting the MP1 neuron and the aCC neuron with Lucifer Yellow and measuring the distance of the growth cone from the neuron cell body. The effects of the 2B2 mAb on neuronal pathfinding were assayed by adding approximately 0.1 mg ml^{-1} 2B2 ascites or affinity-purified 2B2 mAb to the culture media. The 2B2 mAb was affinity purified by passage over a protein A–Sepharose CL-4B column (Pharmacia). Control embryos were cultured either in the absence of antibody or in the presence of 0.1 mg ml^{-1} 13E10 mAb ascites. Extent of axon outgrowth was assayed before and after the culture period by microinjecting the MP1 and the aCC cells with Lucifer Yellow.

Statistical analysis

Differences in distance of axon outgrowth between experimental and control embryos in the antibody-blocking experiments were analyzed using the Mann Whitney U test. This is a nonparametric test of two independent groups, which shows the probability that two groups have been drawn from the same population.

Results

The 2B2 mAb recognizes a nervous-system-specific antigen

Initial screening of the 2B2 monoclonal antibody on grasshopper embryos 40% through embryonic development revealed localization in the brain, optic lobes and segmentally repeated ganglia of the central nervous system (CNS) (Fig. 1A). Labeling with the 2B2 mAb appears on all of the nerve tracts in the central nervous system. These include the longitudinal connectives, the anterior commissure, the posterior commissure, the segmental nerve and the intersegmental nerve (Fig. 1B). In addition, no non-neuronal cells appear to label with the 2B2 mAb (Fig. 1). Using standard staining conditions, no labeling of sensory neurons is seen in the periphery.

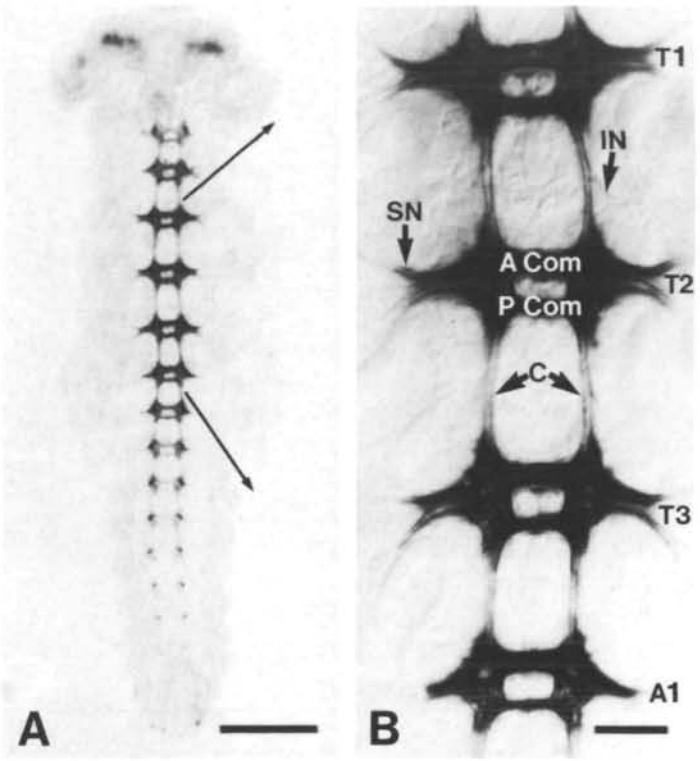

Fig. 1. The 2B2 mAb recognizes the major axon pathways of the CNS in the grasshopper embryo at 37% of development. Whole embryos were labeled with the 2B2 antibody and visualized with a horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody and HRP immunocytochemistry. (A) Low magnification view of the entire embryo. Labeling is restricted to the central nervous system. Arrows indicate region of the CNS shown in B. Scale bar, 500 μm. (B) Higher magnification view of the three thoracic segments (T1, T2, T3) and the first abdominal segment (A1) in another embryo of the same age. Arrows point out the axon tracts of the CNS. SN, segmental nerve; IN, intersegmental nerve; C, longitudinal connectives; A Com, anterior commissure; P Com, posterior commissure. Scale bar, 100 μm.

The expression of the antigen changes during embryogenesis. The earliest labeling is on neuronal processes as the first growth cones are being extended in the CNS at 30% of embryonic development (Fig. 2A). Labeling is seen on axons as fascicles are formed in the CNS (Fig. 2B). At early stages of axonogenesis in the CNS, the most intense labeling with the 2B2 mAb is in the neuropil region where there are many actively extending growth cones (Fig. 2B). When the neuronal scaffold in the CNS is well established, labeling has a uniform appearance on all nerve tracts (Fig. 2C). Labeling of nerve cell bodies is very light from the earliest expression of the antigen through 65% of embryogenesis (Fig. 2A–D, D, arrowhead). At 60% of

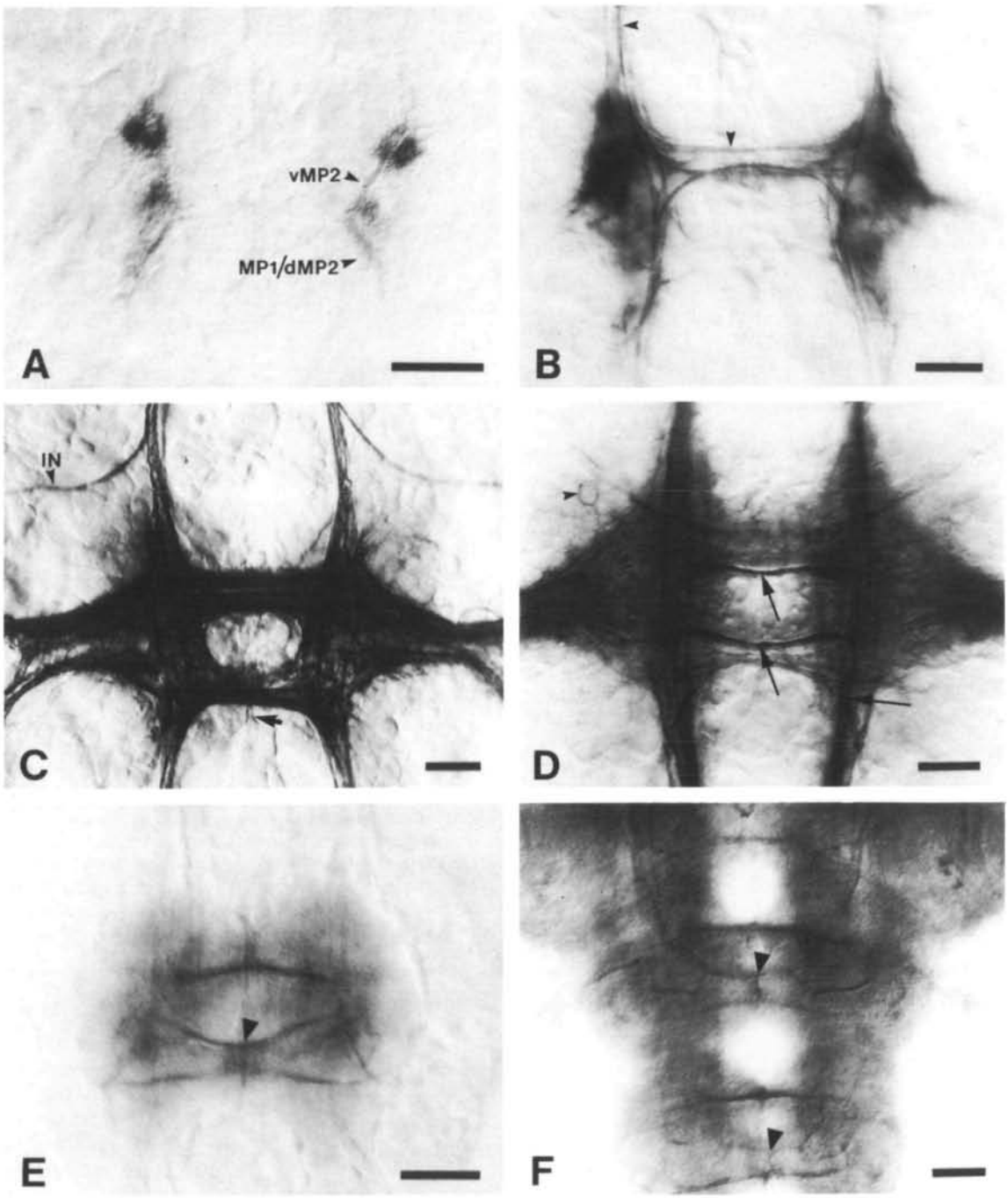

Fig. 2. The labeling pattern with the 2B2 mAb changes during embryonic development. Localization of the 2B2 mAb in the central nervous system was examined by labeling either whole-mount embryos (A, B, C) or dissected nerve cords (D, E, F). Localization was visualized with HRP immunocytochemistry. Dorsal views of a single segment are shown as viewed with Nomarski optics. (A) The 2B2 mAb labels the first processes that extend in the CNS. (B) Labeling is seen on axons as the major nerve tracts are established in the CNS. Horizontal arrowhead points to the developing longitudinal connective tract. Vertical arrowhead points to the anterior commissure. Compare labeling intensities between neuropil region and axon tracts. (C) All of the nerves in the CNS show labeling. For example, arrowhead points to intersegmental nerve and small arrow points to median fiber tract. See also Fig. 1B. (D) At 60% of embryogenesis, labeling appears more strongly on some axon tracts (arrows) as the original labeling pattern begins to fade. Arrowhead points to light cell body labeling. (E) The 2B2 mAb is localized to a restricted subset of axon tracts in ganglia from 70–100% of embryogenesis. (F) View of the third thoracic and the first two abdominal segments in the metathoracic ganglion illustrate that the restricted subset of labeled axon tracts is visible in a segmentally repeated manner. Compare arrowheads with arrowhead in E. The times of embryonic development shown are as follows: (A) 30%, (B) 35%, (C) 45% (D) 60% (E) 90% (F) 80%. Scale bars, 30 μm.

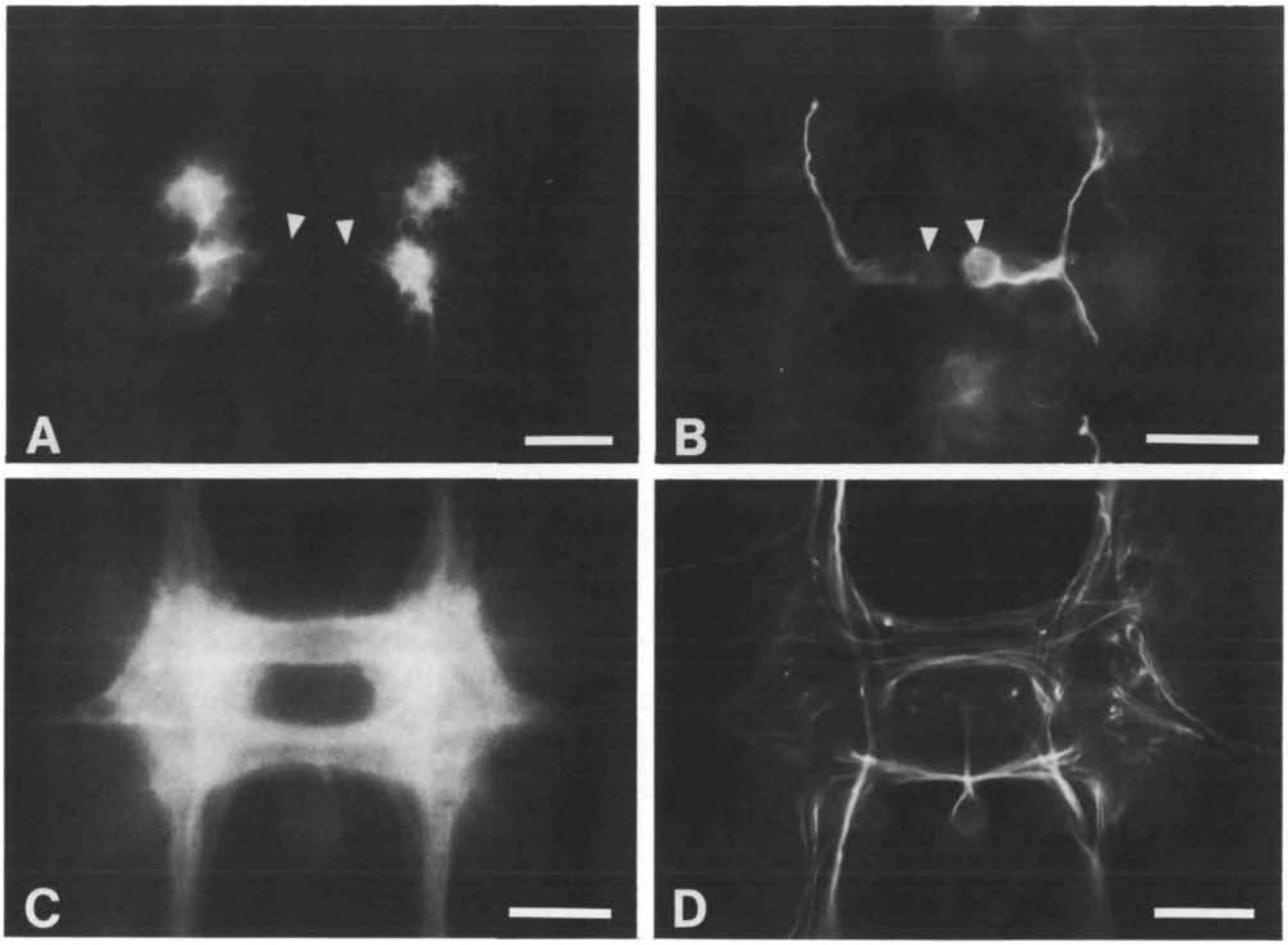

Fig. 3. The 2B2 mAb recognizes elements of the neuropil in addition to axons. Comparison of labeling patterns within a single ganglion of the 2B2 mAb with the 8B7 mAb, which recognizes axons and neuron cell bodies at the stages of development shown. Embryos were labeled with either the 2B2 mAb (A,C) or the 8B7 mAb (B, D). (A, B) Arrowheads point to the MP1 cell bodies. The times of embryonic development shown are as follows: (A) 30 % (B) 32 % (C) 40 % (D) 40 %. A,C are visualized with a fluorescein-conjugated secondary antibody and B,D are visualized with a rhodamine-conjugated secondary antibody. Scale bars, 30 μm.

embryonic development, labeling starts to fade (Fig. 2D). Some axon tracts appear more strongly labeled than others as the majority of axon tracts in the CNS begin to lose the epitope recognized by the 2B2 mAb. By 70 % of embryonic development, the axons recognized by the mAb represent a small subset of tracts in the CNS (Fig. 2E). This pattern is present for the remaining period of embryogenesis and can be seen in- hatchlings. The restricted pattern of labeling is segmentally repeated in the thoracic and abdominal segments of the nerve cord (Fig. 2F). Cryostat sections of adult nerve cords and whole ganglia dissected from adult grasshoppers do not label (data not shown) suggesting that the antigen recognized by the 2B2 mAb is not found in the adult CNS.

The 2B2 mAb recognizes elements of the CNS in addition to axons. The 2B2 mAb labeling pattern was compared with that seen with a monoclonal antibody (8B7 mAb) that recognizes a cytoplasmic epitope in axons and cell bodies at these stages of development (Fig. 3). The labeling with the 2B2 mAb fills the space of the neuropil and has a somewhat fuzzy appearance (Fig. 3A, C) in comparison with the sharp delineation of axons seen when embryos are labeled with the 8B7 mAb (Fig. 3B, D). The extent of labeling within the neuronal scaffold suggests that in addition to axons the 2B2 mAb recognizes growth cones and the many filopodia present along the length of axons. The fuzzy appearance of labeling suggests that the antigen recognized by the 2B2 mAb may be locally secreted.

A more detailed analysis of the labeling pattern was made using electron microscopy. Live 37 % embryos were labeled with the 2B2 mAb and then prepared for microscopy. Labeling is present on surfaces of neuronal processes (Fig. 4). Demonstration that the 2B2 mAb recognizes a surface epitope is also shown by its ability to label live embryos with the same pattern as that seen with fixed tissue. Fig. 4 shows sections of the longitudinal connective (A), posterior commissure (B), segmental nerve (C), and the intersegmental nerve (D) from embryos labeled with the 2B2 mAb. The localization of the 2B2 mAb is similiar in the different nerves

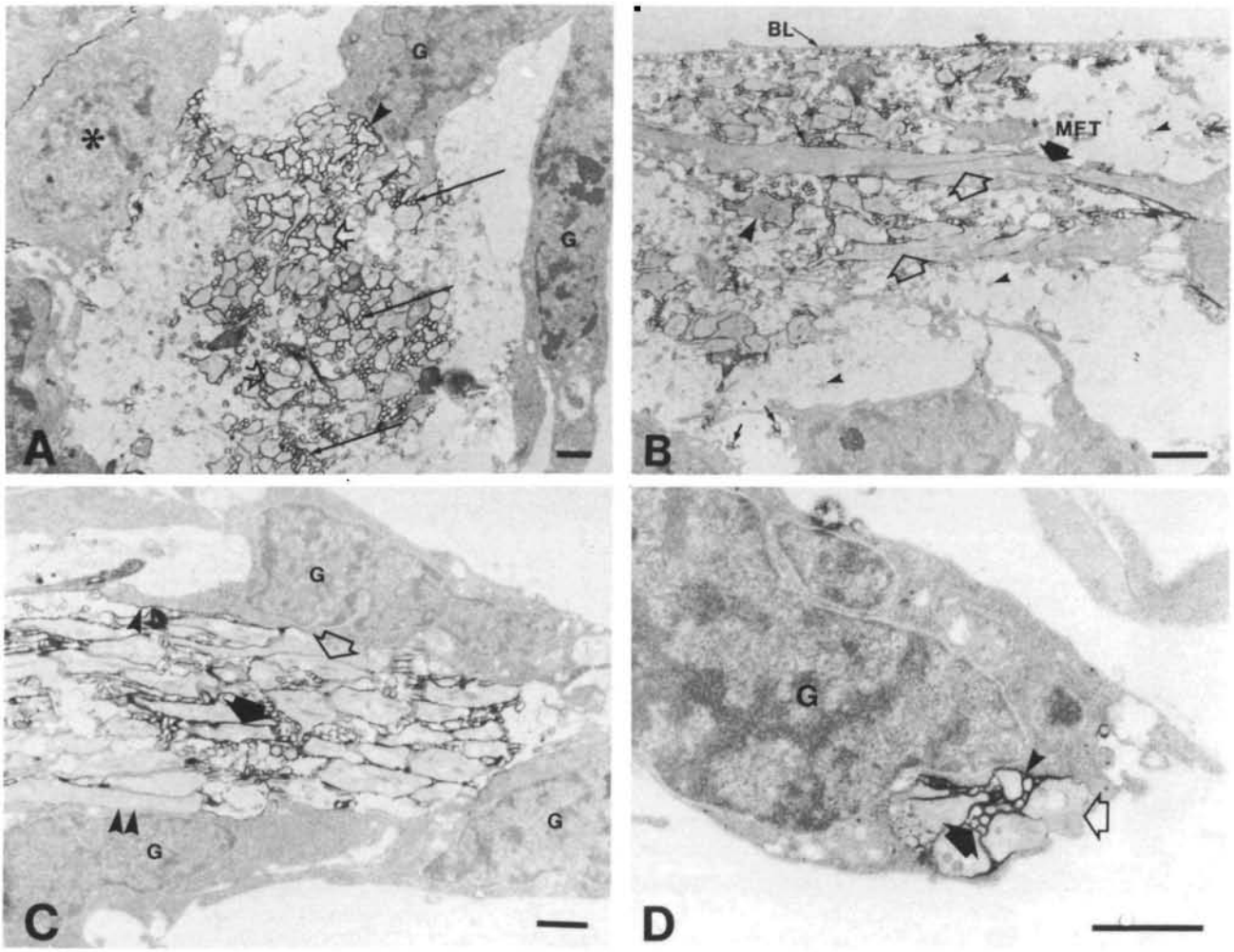

Fig. 4. Immunoelectron microscopy showing the localization of the antigen on membrane surfaces. 36% embryos were labeled with the 2B2 mAb and visualized using horseradish peroxidase (HRP) immunocytochemistry. The localization of the 2B2 mAb is similiar in the different axon tracts examined. (A) Cross section of a connective at the level of the anterior edge of the median neuroblast. Medial is to the right. The most intense labeling is seen on membranes of axons (open arrows) and filopodia (arrows) in regions of contact with neuronal processes. Cell body labeling is not continuous around the entire perimeter of the membrane (asterisk). In addition, faces of cell membranes that contact neuronal processes show labeling (arrowhead). (B) Sagittal section of the posterior commissure at the midline. Posterior is to the right. The median fiber tract (MFT) is lightly labeled. Large arrowhead points to a probable growth cone. Some axons that are in close apposition with other axons are not labeled (open arrows). Many filopodia that lie away from axon tracts and do not contact other processes are not labeled (arrowheads) as compared with filopodia that contact other neuronal processes (small arrows). (C) Transverse section of the segmental nerve. Medial is to the right. Open arrow points out regions of axons in close apposition with another axon that do not show labeling with the 2B2 mAb. An example of the presence of the mAb between two closely apposed axon membranes is shown by a closed arrow. The glial cells that surround the segmental nerve also show mixed labeling; there are both faces that do label (arrowhead) and faces that do not (double arrowhead). (D) Transverse section of the intersegmental nerve. Labeling is seen on faces of glial cell membranes that contact neuronal processes (arrowhead). In the intersegmental nerve, there are examples of unlabeled faces of axons in regions accessible to the 2B2 mAb (open arrow) in addition to labeling on surfaces of axons as seen in other axon tracts (closed arrow) (See also A, B, C). For all plates dorsal is up. G, glial cell; BL, basal lamina. Scale bars, 4 μm.

examined. This is consistent with the uniform labeling seen at the light level at the same stage of development. The antigen is associated with the cell membranes of axons, growth cones and filopodia, and on some neuron cell bodies. Typically, the most intense labeling is seen in regions of contact between neuronal processes (Fig. 4A, open arrows). Cell body labeling is not continuous around the entire surface of cell bodies (Fig. 4A, asterisk). It is seen mostly on faces of the cell membrane that are in contact with filopodia or axons (Fig. 4A, D, arrowheads). Some of these labeled cells appear to be glial cells because they wrap around a nerve and have an irregular-shaped nucleus which is characteristic of glial cell morphology (Hoyle, 1986;

Jacobs and Goodman, 1989). However, not all glial cells that contact axon tracts label with the 2B2 mAb (Fig. 4C, double arrowhead).

The 2B2 mAb is not present over the entire surface of neuronal processes. In contrast with filopodia that contact other neuronal processes (Fig. 4B, small arrows), filopodia that lie far from axon tracts and are not in contact with axons, cell bodies or other filopodia either label lightly or do not label at all (Fig. 4B, arrowheads). Occasionally, labeling is absent along regions of axon membrane in close contact with other axon surfaces (Fig. 4B, C, open arrows). In the same axon tract there are examples of labeling between two other tightly apposed axons (Fig. 4C, closed arrow). In the intersegmental nerve, no labeling can be seen on an axon face that does not appear to be in close apposition with any cell membrane or neuronal process (Fig. 4D, open arrow).

In addition to observations of labeled embryos using Nomarski optics, we examined more directly the question of whether pioneering axons are recognized by the 2B2 mAb. Live embryos were cultured in the presence of the antibody and then specific neurons known to pioneer axonal pathways were filled with Lucifer Yellow. The growth cones of the cells examined do not extend beyond the mAb labeling (data not shown). Both axons that navigate through environments comprised of non-neuronal substrata and axons that encounter other axons in their environment as they navigate express the antigen recognized by the 2B2 mAb.

Biochemical characterization

As a first step in characterizing the antigen recognized by the 2B2 mAb, an immunoprecipitation was performed using iodinated membrane extracts from 40% embryos. Under these conditions the 2B2 mAb precipitates a unique protein band of 160×10^3 M_r (Fig. 5, Lane 1). As a positive control, the 70×10^3 M_r fasciclin I glycoprotein (Bastiani *et al.* 1987) was precipitated using the 3B11 mAb (Fig. 5, Lane 2). A negative control demonstrating nonspecific trapping by the immune complex can be seen in Lane 3 in which membrane extracts were exposed to the 13E10 mAb. This mAb fails to precipitate a unique molecule.

2B2 mAb blocks outgrowth along axonal substrata

We observed the development of the CNS in the presence of the 2B2 mAb to determine if the antigen recognized by the 2B2 mAb has a functional role in neuronal pathfinding. Grasshopper embryos can be cultured outside of the egg for approximately 5–7% of embryonic development. This length of culture period is sufficient to detect effects of the 2B2 mAb on axon outgrowth. Either affinity-purified 2B2 mAb or ascites fluid from a mAb that has no effect on axon outgrowth (13E10 mAb) was added to 33% embryos and the embryos incubated for 3–5% of development. The 13E10 mAb labels all neurons and processes at a greater apparent intensity than the labeling on neurons with the 2B2 Mab. In the initial blocking experiments, ascites fluid of the 2B2 mAb or ascites fluid of the 2B2 mAb that had been heat treated by incubation at 60°C for one hour was used. As a further experimental control, affinity-purified 2B2 mAb was added to the cultured embryos. Ascites fluid, heat-treated ascites fluid, and affinity-purified 2B2 mAb all showed the same effect on growing neurons in cultured embryos.

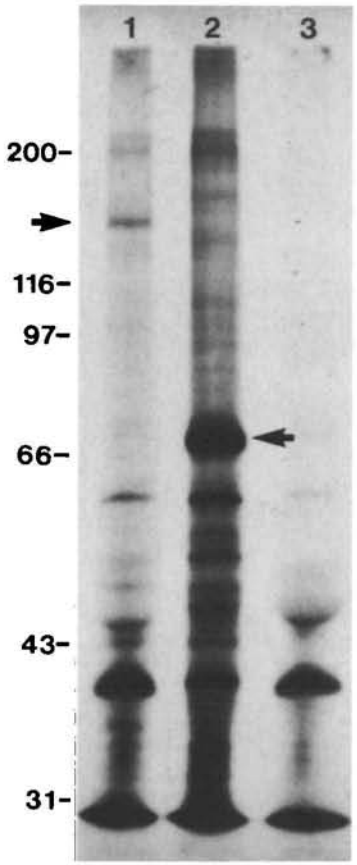

Fig. 5. Immunoprecipitation: Membrane proteins from 40% embryos were labeled with ^{125}I and then solubilized in NP-40. The iodinated proteins were then sequentially exposed to the 3B11 mAb, the 2B2 mAb, and the 13E10 mAb in that order. The precipitated proteins were analyzed by SDS–PAGE on a 7.5% gel under reducing conditions. Lane 1 shows the immunoprecipitation of a specific 160×10^3 M_r band (arrow) using the 2B2 mAb. The precipitation of fasciclin I, a 70×10^3 M_r protein, by the 3B11 mAb serves as a positive control (Lane 2, arrow). The 13E10 mAb does not precipitate a specific band and thus shows nonspecific trapping by the antibody complex (Lane 3).

The formation of the major nerve pathways during development of the CNS is abnormal in the presence of the 2B2 mAb (Fig. 6). One striking anomaly is the difference in the number of neurons in the neuropil region compared with the few neurons that extend through the connective as far as the adjacent segment (Fig. 6, filled arrows). Normally there are many neurons that pass through the length of the connective (Fig. 2C). In addition, the commissural pathways are sometimes malformed by the presence of the

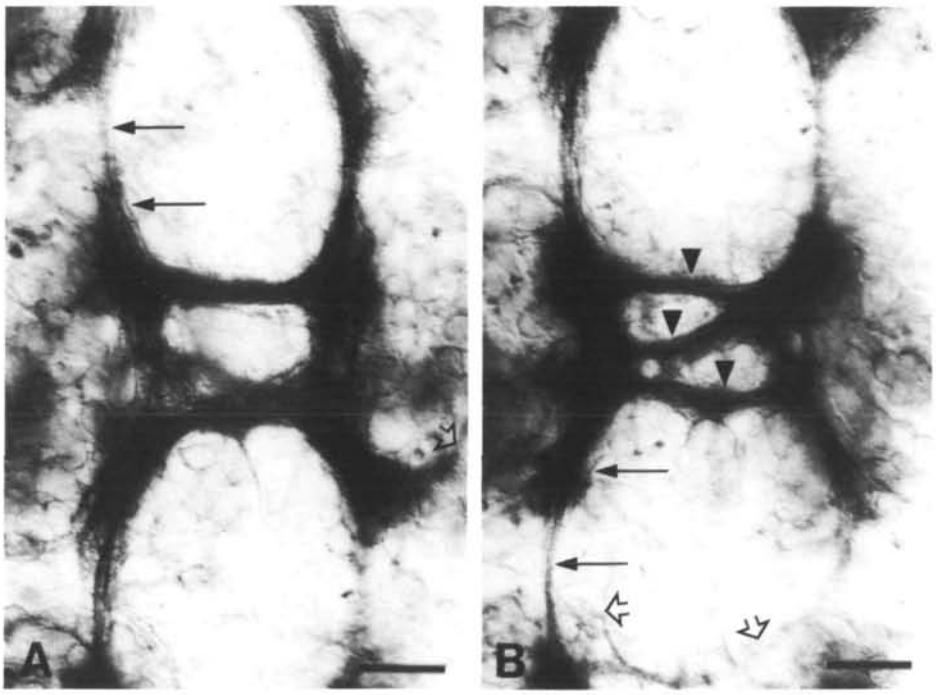

Fig. 6. Embryos cultured in the presence of the 2B2 mAb have an abnormal CNS (A, B). Almost all of the axon outgrowth in the depicted segments has occurred in the presence of the 2B2 mAb. Connectives appear thinner than normal as if growth cones are unable to extend out of the neuropil (filled arrows). In some cases, the commissures are malformed (arrowheads in B). In addition, several neurons have extended along aberrant pathways, visible as neurons present outside of the normal neural scaffold (open arrows). Scale bar, 30 μm.

2B2 mAb (Fig. 6, arrowheads). Another example of abnormalities in the central nervous system due to presence of the 2B2 mAb is the formation of many aberrant pathways (Fig. 6, open arrows).

To examine more closely the effects of the 2B2 mAb on neuronal pathfinding, we observed axon outgrowth of identified neurons in the presence of the 2B2 mAb. We chose to assay the MP1 and aCC neurons. The MP1 neuron extends an axon in an environment devoid of other axons and pioneers a longitudinal pathway in the connective (Goodman *et al.* 1982). When the MP1 growth cone reaches the next posterior segment, it fasciculates with and continues to grow along the axon of its homologue from that segment. We assayed the MP1 cell before it began to grow on an axonal substratum. The aCC cell extends its growth cone along the surface of pre-existing axon tracts during the course of its navigation (du Lac *et al.* 1986). It initially sends a process posteriorly along the U fascicle longitudinal tract and then turns laterally at the segment boundary cell and follows the U fascicle along the intersegmental nerve (Fig. 7A).

Axon length at the start of the culture period was determined in several embryos from the same clutch as those used for the experiment. The MP1 and the aCC neurons were microinjected with Lucifer Yellow and the distance from the growth cone to the cell body was measured. After allowing embryos to develop in culture, axon length of the MP1 and the aCC neuron were again assayed by filling the cells with Lucifer Yellow. The degree of axonal outgrowth over the course of the culture period was compared between embryos incubated in the presence of the 2B2 mAb and control embryos. Axons in embryos cultured in the absence of antibody or in the presence of the control 13E10 mAb grew the same distance during the culture period.

Neurons that grow on axonal substrata in embryos cultured with the 2B2 mAb are specifically blocked in axon outgrowth. When the aCC neuron was assayed in an embryo cultured in the presence the 2B2 mAb, its outgrowth was almost completely blocked as compared to outgrowth in control embryos (compare Fig. 7A and 7B). Fig. 8A shows mean values of axonal outgrowth during the culture period. Outgrowth of the aCC cell was examined in three different segments of the grasshopper embryos: the second and third thoracic (T2 and T3) and the first abdominal segment (A1). The nervous system matures in an anterior-to-posterior fashion. In this way, we could look for effects of the 2B2 antibody at different time points during the navigation of the aCC growth cone. In all three segments examined, the aCC neuron growing in the presence of the 2B2 mAb grew less than in the control embryos. The further the neurons in the control embryos had grown during the culture period, the greater the difference in outgrowth between cells in the experimental and control embryos. The effect of the 2B2 mAb on axon outgrowth is concentration dependent. When the concentration of the 2B2 mAb added to cultured embryos is lowered to 1/10th of the concentration used in the experiment, growth cones are partially blocked in their degree of outgrowth; when the concentration of the 2B2 mAb is 1/100th of the original concentration, axon outgrowth is not affected.

Even in the presence of the 2B2 mAb, a few aCC cells continued to extend neuronal processes (Fig. 7D). One exception grew as far as did the aCC cell in control embryos. However, examination of the morphology of this neuron reveals that it grew along an aberrant pathway. In Fig. 7D, the aCC growth cone continued to extend in a posterior direction past where it would normally turn laterally towards the body wall. This

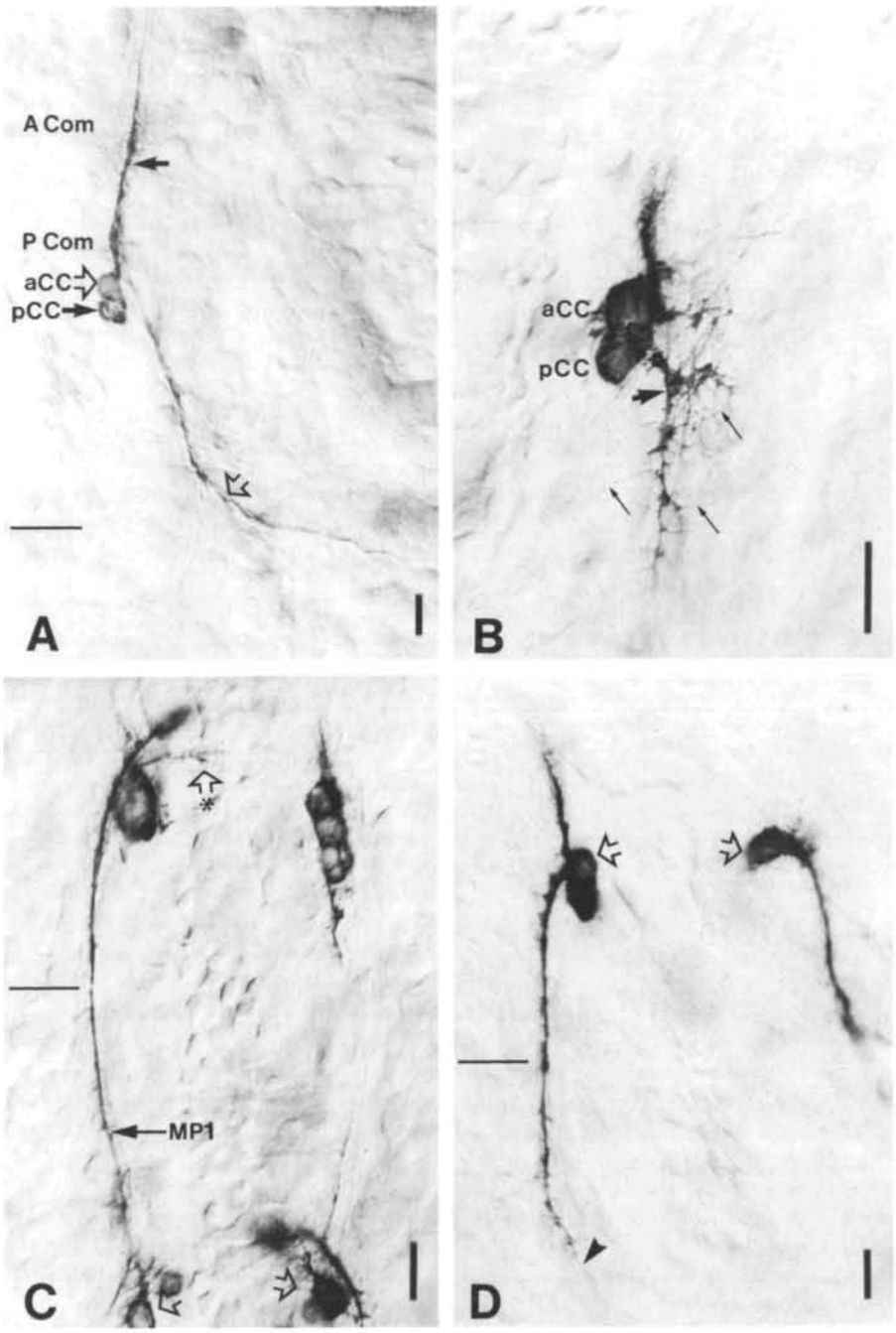

Fig. 7. The 2B2 mAb specifically alters outgrowth of growth cones on neuronal substrata. Embryos were cultured in the presence of approximately 0.1 mg ml^{-1} affinity-purified 2B2 mAb for 24 h (see Methods). Cells were injected before and after the culture period and axon outgrowth was compared between the two assay times. (A) The extent of growth of the aCC in a control embryo which was cultured in the presence of the 13E10 mAb. (B) The same cells as shown in A but the embryo was cultured in the presence of the 2B2 mAb. The growth cone has hardly extended from the cell body of the aCC cell. The greater magnification of panel B (compare with A) reveals the numerous filopodia extending from the aCC cell growth cone (small arrows). (C) This embryo shows both the MP1 neuron (cell body is out of focus) that grows on non-neuronal substrata and the aCC cell (open arrows) in an embryo that has been cultured with the 2B2 mAb. Outgrowth of the MP1 axon (arrow) on basal lamina was not blocked by culturing embryos with the 2B2 mAb. Arrow with asterisk points to the growth cone of an aCC neuron which has made an aberrant pathway choice. The growth cone of this cell has extended across the posterior commissure almost to the midline. (D) An example of an aCC cell axon that has extended along an aberrant pathway (open arrow, left side). On one side of the embryo, the aCC cell has been blocked in its outgrowth. On the contralateral side, the same cell continued to extend in a posterior direction approximately 60 μm past where it would normally turn laterally at the segment boundary. (Compare segment boundary bar with arrowhead). Note: the blocking experiment in D was started at an older age than that of A, B, and C. Scale bars, 20 μm.

aberrant cell extended approximately 60 μm past its contralateral homologue cell in the same segment whose growth was blocked by the 2B2 mAb (compare open arrows). Another example of an aberrant pathway choice made by the aCC is shown in Fig. 7C (open arrow with asterisk) in which the aCC cell extended a process a short distance across the posterior commissure instead of along the U fascicle in a posterior direction. The aberrant pathway choices made by the aCC growth cone in the presence of the 2B2 mAb are similiar to the behavior of the aCC neuron when its normal axonal substrata are experimentally or naturally altered (see Discussion).

The growth cones of the aCC cells in embryos cultured in the presence of the 2B2 mAb are large and elaborate. The number and length of filopodia is often striking. This morphology demonstrates that the growth cones do not die or retract in the presence of the antibody. Although the 2B2 mAb blocks axonal extension, it does not block filopodial extension (Fig. 7B, small arrows).

The outgrowth of the MP1 cell during the culture period was unaffected by addition of the 2B2 mAb. In embryos cultured in the presence of the 2B2 mAb, the MP1 neuron extended a process as far as did the MP1 cell in control embryos cultured in the presence of the

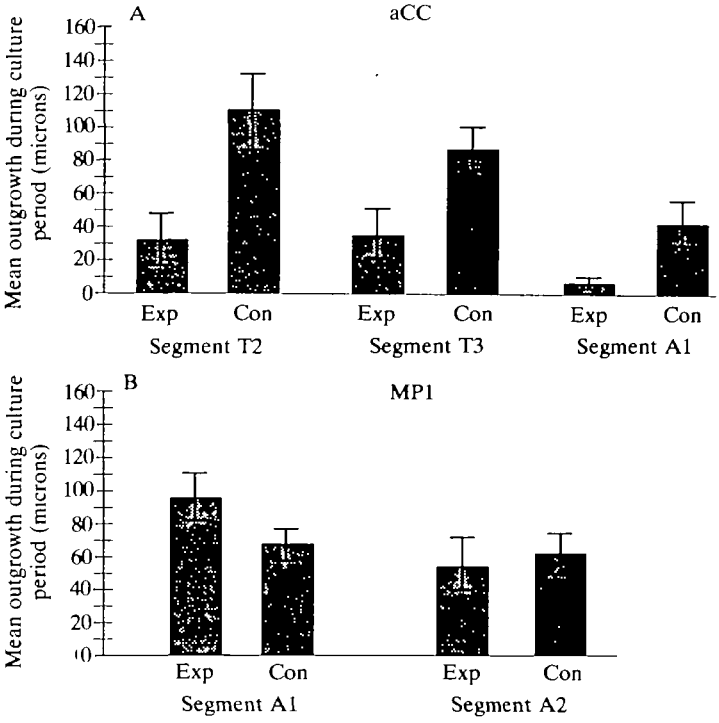

Fig. 8. Mean outgrowth of the aCC (A) and the MP1 (B) neurons in cultured embryos. The growth of axons was compared between embryos cultured in the presence of the 2B2 mAb (Exp) and those cultured in the presence of the 13E10 mAb (Con). Both the MP1 and the aCC neurons were assayed in several segments in the embryo. Each segment represents a different developmental time point of axon outgrowth. Comparisons of distance of axon growth during the culture period are made between embryos in equivalent segments. Standard deviation from the mean for each set of data is shown by an error bar.

13E10 mAb or in the absence of a mAb (see Fig. 8B). The fact that the MP1 is unaffected by the addition of the 2B2 mAb allows it to serve as a good internal control for nonspecific effects on axon outgrowth due to the presence of the 2B2 mAb. In many cases, we assayed both the MP1 cell and the aCC cell in the same embryo. In Fig. 7C, the MP1 neuron has extended a longitudinal process into the next posterior segment (short arrow). In the same embryo, the aCC growth cone either stopped growing or grew along an aberrant pathway (Fig. 7C, open arrows).

A quantitative analysis of the antibody-blocking experiment was undertaken to determine if the differences observed in axon outgrowth during the culture period between the experimental and control embryos were statistically significant (Table 1). The probability that the length of axon outgrowth for the aCC neuron in control embryos is the same as that in experimental embryos is <0.001 in all segments examined. In contrast, the lengths of axons in the MP1 cell in experimental and control embryos are not significantly different in both the segments examined.

Discussion

In this paper, we describe a nervous-system-specific antigen recognized by the 2B2 mAb that shows a change in expression during the course of embryonic development in the grasshopper. The 2B2 mAb specifically precipitates a molecule of 160×10^3 M_r. The antigen is expressed on the surface of axons, growth cones, filopodia and cell bodies. During the stages of embryogenesis when the mAb labels all axons, individual axons are not visible because the labeling fills the neuropil region. One possibility to explain this observation is that the labeling on the numerous filopodia along the length of axons fill in the space between axons and obstruct a clear view of individual axon tracts. Additionally, a locally secreted form of the antigen may exist. In the case of axonin-1, a protein isolated from chick that labels nerve fiber tracts, there is both a secreted form and an integral membrane form (Ruegg *et al.* 1989). The secreted form is not detected by immunoelectron microscopy. This is similiar to our electron microscopy observations in which the 2B2 mAb is localized to membrane surfaces and no secreted form of the antigen is detectable. The cell body labeling is seen primarily along faces of the cell membranes that are in contact with neuronal cell processes. In addition to localization of the 2B2 mAb on surfaces of neuron cell bodies, electron microscopy reveals labeling on glial cells. The question remains open as to whether glial cells actually express the antigen recognized by the 2B2 mAb or whether the antigen is expressed only by

Table 1. *Axon outgrowth of neurons cultured in the presence or absence of the 2B2 mAb*

Cell	Segment	Sample	Mean	S.D.	*N*	Range	U value	*P*
aCC	T3	Exp	51.3	29.7	15	1–120	140	<0.001
aCC	T2	Con	129.0	44.5	10	60–180		
aCC	T3	Exp	38.0	28.4	15	0.5–100	137.5	<0.001
aCC	T3	Con	89.5	24.81	10	58–122		
aCC	A1	Exp	8.5	5.97	14	0.5–17	134	<0.001
aCC	A1	Con	43.5	25.03	10	5–75		
MP1	A1	Exp	151.7	27.54	3	125–180	2.5	n.s.
MP1	A1	Con	124.4	18.08	5	100–150		
MP1	A2	Exp	98.6	31.6	9	45–150	46.5	n.s.
MP1	A2	Con	106.22	24.1	9	65–150		

The statistical test used for analysis of length of axon outgrowth is the Mann Whitney U test.

neurons and can diffuse and bind to a ligand on the glial cell membrane.

Some nonuniform labeling on neuronal processes is observed with electron microscopy although no entire axon fascicle or tract was found to be unlabeled. Unlabeled filopodia do not contact other neuronal processes. This observation is consistent with an antigen that functions in regions of contact between neuronal processes. Another example where the antigen recognized by the 2B2 mAb is absent from neuronal processes is on some areas of axon surfaces. This may be due to the tissue being inaccessible to the 2B2 mAb since many of the unlabeled axonal surfaces are in regions of tight axon–axon contact. In the intersegmental nerve, there is no labeling on an axon face that does not appear to be in close apposition with any cell or neuronal process. Examination of a single EM section does not allow a definitive conclusion as to the environment around an axon tract. Areas that appear to be unapposed by other tissue may actually be within enclosed spaces that are inaccessible to the 2B2 mAb. From the present data, one cannot distinguish between the possibilities that some regions of neuronal processes in the CNS do not express the antigen recognized by the 2B2 mAb or that lack of labeling along some regions of axons is a result of the 2B2 mAb not having access to cell surfaces of all neurons in the embryo.

The 2B2 mAb appears to label all axons in early stages of embryogenesis, but by 70% of embryonic development the labeling pattern is restricted to a small subset of axon tracts in the ganglia. The identity of this subset of axon fascicles is unknown. It has been documented in the locust that there are a small number of neuronal precursor cells, or neuroblasts, which continue to produce progeny actively through most of embryogenesis (Shepherd and Bate, 1990). Actively dividing neuroblasts are present until 70% of embryogenesis in the unfused abdominal ganglia and until 92% in the thoracic ganglia. Thus, it is possible that the axon tracts that label during later stages of embryogenesis represent fascicles upon which neurons born during the later half of embryonic development are extending processes. Alternatively, the mAb may recognize an epitope common to more than one molecule: one whose pattern of expression correlates with axonogenesis and another which is expressed during later stages of embryogenesis. It is also possible that the antigen is a single molecule with multiple functions.

The antigen recognized by the 2B2 mAb has an interesting distribution compared with that of other molecules identified in the nervous system of invertebrates. Other molecules that label axons in grasshopper, such as fasciclin I and II, are expressed in many tissues outside of the nervous system while the antigen recognized by the 2B2 mAb is confined to the central nervous system. Moreover, the most prevalent expression of the antigen correlates with the period in embryogenesis when the majority of axon outgrowth occurs. The specific distribution of the antigen on axons suggests that its function is unique to the nervous system during the process of axon outgrowth as opposed to the birth of neurons, dendritic outgrowth or synaptogenesis. To learn more about the antigen recognized by the 2B2 mAb, we are currently trying to purify the antigen and identify the gene that encodes the antigen by immunoscreening an expression library.

The formation of the major nerve pathways is abnormal in the presence of the 2B2 mAb. To examine more closely the effects of the 2B2 mAb on neuronal pathfinding, we chose to assay the MP1 and the aCC cells because we wanted to examine the effect of the 2B2 mAb on neurons extending processes in different types of environments, namely along non-neuronal substrata as opposed to along other axons. From our examination of the aCC cell, we infer that the 2B2 mAb blocks growth of neurons that are extending growth cones along other axons. In contrast, the growth of neurons that normally extend on non-neuronal substrata such as basal lamina and glial cells are unaffected when cultured in the presence of the 2B2 mAb. These results demonstrate that the antigen recognized by the 2B2 mAb may be important for neuron–neuron interactions, i.e. growth cones extending along other axons. The behavior of the aCC growth cone when blocked by the 2B2 mAb is the same as when the axon substratum upon which it grows is absent. The aCC growth cone normally extends along the U neurons. If the U neurons are ablated before the aCC neuron extends a growth cone, the aCC growth cone points anteriorly without extending an axon (du Lac *et al.* 1986). Similarly, if the 2B2 mAb is added to the cultured embryo before the aCC has extended an axon, its growth cone remains close to the cell body pointing in an anterior direction.

An interesting observation from the antibody-blocking experiments is that the few aCC neurons that were not blocked in growth in the presence of the 2B2 mAb made aberrant pathway choices. These aCC cells appear to extend growth cones only within the neuronal scaffold in the CNS rather than extending processes into regions lacking axons. The specific behavior exhibited by these unblocked aCC growth cones is similiar to behavior of the aCC neuron when the substrata upon which it grows are altered. For example, one of the aCC neurons that made an aberrant pathway choice in the presence of the 2B2 mAb continued to extend a growth cone posteriorly beyond where it normally turns onto the intersegmental nerve. When the segment boundary cell (SBC), an early glial cell, is ablated, the aCC growth cone either stops growing at the level of the intersegmental nerve or continues to extend posteriorly along a longitudinal pathway (Bastiani and Goodman, 1986*b*). The segment boundary cell is located at the junction between the longitudinal connective and the intersegmental nerve. The presence of the SBC is necessary for the initial formation of the intersegmental nerve. Another of the aCC neurons that made an aberrant pathway choice grew along the posterior commissure. Interestingly, the aCC neuron in the environment of the first subesophageal segment grows across the posterior commissure (Bastiani and

Seaver, personal observation). Thus, when addition of the 2B2 mAb does not block growth of the aCC growth cone, the aCC neuron behaves as if its environment is altered such that specific guidance cues are no longer available to allow the growth cone to make the correct pathway choice.

Although the growth cones of neurons blocked in outgrowth by addition of the 2B2 mAb have stopped extending, there are extensive filopodial arborizations. Thus, the 2B2 mAb does not block growth of an axon by causing either the collapse or death of the growth cone. The complex growth cone morphology observed in the presence of the 2B2 mAb is reminiscent of a growth cone actively exploring its environment (Raper *et al.* 1983*a*); the growth cone has a spread out appearance and extends numerous filopodia in multiple directions. Harrelson and Goodman (1988) observed similar complex growth cone morphology in neurons stalled in outgrowth by antibodies against fasciclin II.

The outgrowth of the MP1 cell is not blocked by the presence of the 2B2 mAb. However, the MP1 cell does express the antigen recognized by the 2B2 mAb. Does the antigen function in navigation of the MP1 cell as it travels through a non-neuronal environment? An explanation more consistent with our experimental observations is that the antigen recognized by the 2B2 mAb is expressed on the surface of the MP1 cell in preparation for other axons that will later grow along its surface. Moreover, the MP1 cell may utilize the antigen recognized by the 2B2 mAb for its own pathfinding. When the MP1 growth cone reaches the next posterior segment, it fasciculates with and continues to grow along the axon of its homologue from that segment. The antigen recognized by the 2B2 mAb may be important for the axon–axon interactions that occur as the MP1 cell extends along the MP fascicle.

The effect on growth of the aCC and MP1 neurons reflects the overall appearance of a segment that has developed in the presence of the 2B2 mAb. Several aspects of the CNS appear abnormal suggesting that the growth of many neurons are affected by the presence of the 2B2 mAb. Not all axon outgrowth in the segment is blocked in the presence of the 2B2 mAb. This is consistent with the fact that the growth of the MP1 neuron is unaffected and that the growth cones of the aCC extend a short distance in the presence of the 2B2 mAb. There are several examples of aberrant pathway choices made within a segment (Fig. 6) indicating that the pathway choice of several neurons is influenced by the presence of the 2B2 mAb. A definitive conclusion of whether all neurons that grow along axonal substrata are blocked in their outgrowth by the presence of the 2B2 mAb is not possible from the experiments presented. Our experiments support the idea that axon outgrowth in the presence of the 2B2 mAb affects many neurons of the CNS.

The antigen recognized by the 2B2 mAb does not show a distribution that can obviously provide specific positional information to a growth cone. The presence of this antigen may allow a growth cone to distinguish between axonal substrata and non-axonal substrata and may be important in supporting neuron outgrowth along other axons. With the antigen recognized by the 2B2 mAb present on the surfaces of axons, growth cones can grow along axons and utilize molecular cues that distinguish axonal pathways. The fact that (1) the antigen recognized by the 2B2 mAb is nervous system specific and is expressed during axonogenesis and (2) addition of the 2B2 mAb specifically blocks growth or causes aberrant pathway choices of growth cones on axonal substrata, supports the idea that this antigen plays a role in the establishment of the axon tracts in the CNS of grasshoppers.

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