Cerebellar Granule Cells Contain a Membrane Mitogen for Cultured Schwann Cells

Patrick W. Mason, John W. Bigbee,* and George H. DeVries

Department of Biochemistry and Molecular Biophysics, and *Department of Anatomy, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Abstract. Proliferation of Schwann cells is one of the first events that occurs after contact with a growing axon. To further define the distribution and properties of this axonal mitogen, we have (a) cocultured cerebellar granule cells, which lack glial ensheathment in vivo with Schwann cells; and (b) exposed Schwann cell cultures to isolated granule cell membranes. Schwann cells cocultured with granule cells had a 30-fold increase in the labeling index over Schwann cells cultured alone, suggesting that the mitogen is located on the granule cell surface. Inhibition of granule cell proteoglycan synthesis caused a decrease in the granule cells' ability to stimulate Schwann cell proliferation. Membranes isolated from cerebellar granule cells when added to Schwann cell cultures caused a 45-fold stimulation in [^{3}H]thymidine incorporation. The granule cell mitogenic signal was heat and trypsin sensitive and did not require lysosomal processing by Schwann cells to elicit its proliferative effect. The ability of granule cells and their isolated membranes to stimulate Schwann cell proliferation suggests that the mitogenic signal for Schwann cells is a ubiquitous factor present on all axons regardless of their ultimate state of glial ensheathment.

AXONS can exist in vivo in three states of glial ensheathment: glial ensheathed and myelinated, gilal ensheathed but unmyelinated, and nonglial ensheathed. To determine the molecular nature of the signals that influence neuron–glial interactions, investigators have examined the early events that occur after a glial cell contacts an axon. These studies have indicated that one of the first responses that the peripheral nervous system glial cell, the Schwann cell, undergoes upon contact with an axon is a wave of proliferation (1, 2, 27) and the proliferative signal appears to reside on the axon plasma membrane. The stimulatory effect can be duplicated in vitro using either mixed Schwann cell and neuronal cultures (29) or Schwann cell cultures with exogenously added axonal membranes (7, 21). These in vitro studies of the Schwann cell mitogen have indicated that the proliferative signal is a heat- and trypsin-sensitive molecule (7). Axolemmal membranes when added to Schwann cells remain on the outside of the cell (14, 24) and the mitogenic effect of the membranes is not lost upon inhibition of lysosomal activity with NH_4Cl (31) further suggesting that the mitogenic signal requires cell–cell contact. Ratner et al. (18) has shown that inhibition of proteoglycan synthesis of cultured dorsal root ganglion neurons removes the mitogenic potential of the nerve cells. These studies suggest that the mitogenic signal may be a polypeptide that is associated with the membrane by a proteoglycan.

Present knowledge of the Schwann cell mitogen has been determined using axons that will be ensheathed by either oligodendrocytes or Schwann cells. The ability of axons that will never be ensheathed by a myelin-competent glial cell to stimulate glial cell mitosis has not been investigated. The normally nonmyelinated and nonglial ensheathed granule cells of the cerebellum provide the opportunity to examine whether the Schwann cell mitogen is present only on axons that are glial cell ensheathed. The distribution of the Schwann cell mitogen could begin to address the question of what signals might be important during the early events that occur after neuron–glial contact.

We have examined the effect of intact granule cells and isolated granule cell membranes on Schwann cell proliferation. Granule cells contain a mitogen for Schwann cells that appears to be similar to the mitogen found on glial-ensheathed axons in its response to chemical treatment and its sensitivity to the inhibition of proteoglycan synthesis. The presence of the mitogenic signal on an axon that normally does not associate with a myelin-competent glial cell suggests that the presence of the mitogenic signal on an axon is not dependent on the extent of glial ensheathment.

Materials and Methods

Media

Granule cells were plated and maintained in DME (Gibco Laboratories, Grand Island, NY) with 10% type IV Nu-Serum (Collaborative Research Laboratories, Lexington, MA), 25 mM KCl, and 0.6% added glucose. Schwann cells were grown in DME with 10% FCS (Hyclone Laboratories, Logan, UT). Astrocyte-plating media contains DME and Ham's F-12 (Gibco Laboratories) media (1:1) with 10% FCS and 0.5% glucose (wt/vol). Saline 1 is a balanced salt solution containing 138 mM NaCl, 5.4 mM KCl, 1.1

mM Na_2HPO_4, 1.1 mM KH_2PO_4, and 22 mM dextrose, pH 7.0. Homogenizing media contains 10% sucrose, 10 mM TES, pH 7.4, and 0.02% sodium azide. HF media contains DME and 10% FCS.

Preparation of Cultured Granule Cells

Monolayer cultures of cerebellar granule cells were prepared using several modifications of the procedures described by Meier et al. (15) and Wilkin (28). Briefly, the cerebella from 8-d-old rats were removed and treated with 0.025% trypsin in Saline 1 for 15 min at 37°C. An equal volume of 0.004% DNase 1 in Saline 1 was added and incubated at 37°C until the tissue no longer floated in the solution (~1 min). The tissue was quickly centrifuged (50 g for 5 s), the supernatant removed, and fresh DNase 1 was added for an additional 5 min. The tissue was disrupted by triturating through a Pasteur pipette and filtered through a 209-μm Nitex filter (Tetko Inc., Elmsford, NY). The cells were collected by centrifugation at 800 g for 5 min, plated on poly-L-lysine-coated (30) 100-mm glass petri dishes in granule cell media, and incubated at 37°C with 5% CO_2. The antimitotic agent, fluorodeoxyuridine (80 μM) was added 24 h after initial plating to eliminate rapidly dividing, nonneuronal cells. After 72 h, fresh media was added and the cells were allowed to recover for another 96 h.

Preparation of Cultured Schwann Cells

Schwann cells were prepared from 2-d-old rat pups by the method of Brockes et al. (4) as modified by Meador-Woodruff (14). Briefly, sciatic nerves were removed from 2-d-old rat pups and treated with trypsin and collagenase. The mixture was triturated with a Pasteur pipette and filtered through a 209-μm Nitex filter. Cells were suspended in 10 ml of Schwann cell media and plated at a density of 3-5 × 10⁶ cells/10-cm dish. The antimitotic drug cytosine arabinoside (10 μM) was added 24 h after the dissection to reduce the number of rapidly dividing fibroblasts. To eliminate any fibroblasts that survived the cytosine arabinoside treatment, the cultures were treated with anti-Thy 1.1 supernatant from the TIB 103 cell line (American Type Culture Collection, Rockville, MD) and a rabbit complement (Cappel Laboratories, Inc., Cochranville, PA) as previously described (14).

Preparation of Granule Cell-enriched Membranes

8 d after initial plating, granule cells were removed from their culture dishes in a small volume of homogenizing buffer by gentle scraping using a rubber-tipped spatula. The cells were homogenized using 10 strokes of the "A" pestle in a 15-ml Dounce homogenizer (Kontes Glass Co., Vineland, NJ) and the membranes were collected by centrifugation at 82,500 g for 1 h. The membranes were suspended in sterile Saline 1 and the protein concentration was determined using the Bradford dye binding assay (3).

Preparation of Astrocytic Membranes

Astrocytes were isolated and grown in culture using the method of McCarthy and DeVellis (13). Briefly, the cerebra was removed from 2-d-old rat pups and treated with 0.25% trypsin and 0.4% DNase 1 in Saline 1 for 30 min at 37°C. The cells were disrupted by trituration and filtered through a 130-μm Nitex filter. The cells were collected by centrifugation and plated on poly-L-lysine-coated (30) 75-cm² flasks in astrocyte-plating media at a low cell density (4 × 10⁵ cells/ml). Oligodendrocytes were removed by shaking 7 d after dissection. Astrocytes remained in culture for 2 d after shaking at which time they were washed, removed from the flask by scraping using a rubber-tipped spatula, and homogenized with 10 strokes of the "A" pestle in a 15-ml glass Dounce homogenizer in homogenizing media. The membranes were collected by centrifugation at 82,500 g for 1 h. The membranes were suspended in sterile Saline 1, and the amount of protein determined by the Bradford dye binding assay (3).

Addition of Membranes to Purified Schwann Cell Cultures

Schwann cells were plated on 96-well microtiter plates at a cell density of 8,000 cells per well. 24 h after plating, granule cell membranes were added at concentrations of 5, 10, 20, 50, 100, or 200 μg of protein/ml. To exclude the possibility that the mitogenic stimulation was due to astrocytes, which are also present in the cultures, astrocytic membranes were added to Schwann cell cultures at concentrations of 5, 10, 20, 50, 100, or 200 μg of protein/ml. Membranes were incubated with the Schwann cell cultures for 72 h with 0.3 μCi of [³H]thymidine being added for the last 24 h of the experiment.

To determine the extent of [³H]thymidine incorporation, Schwann cells were removed with 0.005% trypsin and 0.02% EDTA, collected on filters using a Titertek (Flow Labs Inc., McClean, VA) cell harvester, and counted in a liquid scintillation counter as described by Yoshino et al. (31).

Heat and Trypsin Treatment of Granule Cell Membranes

Granule cell membranes were used at a concentration of 50 μg/ml for each membrane treatment. Granule cell membranes were heated to 100°C for 10 min, collected by centrifugation, suspended in fresh media, and added to Schwann cells. Granule cell membranes were treated with 0.5 mg/ml of trypsin, type IX (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C. Trypsin treatment was terminated by the addition of 4 mg/ml soy bean trypsin inhibitor, followed by centrifugation. The membranes were suspended in fresh media and added to Schwann cells plated in 96-well microtiter plates. Membranes were added for 48 h at which time 0.3 μCi of [³H]thymidine was added for an additional 24 h. The extent of [³H]thymidine incorporation was determined by the cell harvester assay as described above.

The Effect of Lysosomal Process Inhibition by NH₄Cl on Granule Cell Membrane Mitogenicity

Granule cell membranes were added to Schwann cells at a concentration of 50 μg of protein/ml in the presence of 9 mM NH_4Cl. Schwann cells were plated in 96-well microtiter plates 24 h before the addition of the membranes. Membranes were added for 72 h with 0.3 μCi of [³H]thymidine being added for the last 24 h of the experiment. The extent of [³H]thymidine incorporation was determined by the cell harvester assay as described above.

Granule Cell and Schwann Cell Cocultures

Granule cells were isolated from 8-d-old rat pups as described above, plated on 13-mm glass coverslips at a cell density of 500,000 cells/coverslip, and maintained in culture for 8 d. Schwann cells, which had been treated as described above, were added to the granule cell cultures at a density of 40,000 cells/coverslip. Cells were cocultured for 48 or 72 h with 1.5 μCi of [³H]thymidine being added for the last 24 h of the experiment. Coculture experiments were terminated by removing the media and washing the cultures three times with Saline 1. The cells were fixed with 4% paraformaldehyde in granule cell media without Nu-serum and processed for autoradiography as previously described (6). A minimum of 1,000 cells were counted per coverslip and a labeling index was determined by dividing the number of labeled Schwann cells by the total number of Schwann cells.

Proteoglycan Synthesis Inhibition of Granule Cell and Schwann Cell Cocultures

Granule cells and Schwann cells were isolated and cocultured as described above. 48 h after the addition of Schwann cells, the media was removed and either media alone, media containing 1 mM of 4-methylumbelliferyl-β-D-xyloside, or 1 mM of 4-methylumbelliferyl-α-L-arabinoside, a noninhibitory structural analogue of the β-D-xyloside (18), was added. The cells were incubated for 96 h with [³H]thymidine being added for the last 24 h of the experiment. A labeling index was determined by autoradiography as described above.

Results

Stimulation of Schwann Cell Proliferation by Granule Cell Membranes

Schwann cells were incubated with increasing concentrations of granule cell membranes to determine if the granule cell membranes have the capacity to induce a proliferative response in cultured Schwann cells. Initially, low levels of [³H]thymidine incorporation were seen at granule cell membrane concentrations up to 50 μg/ml (Fig. 1). The amount of stimulation increases in a dose-dependent manner, reaching a maximum incorporation 45-fold over control cells at a membrane concentration of 200 μg/ml. One possible explanation for the observed mitogenicity is that the mitogen
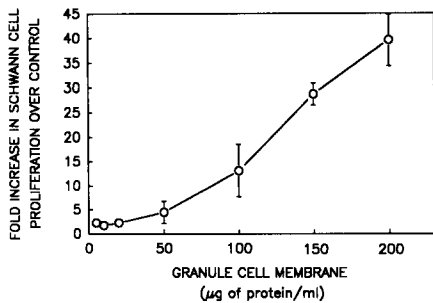
Figure 1. Granule cell membrane stimulation of Schwann cell proliferation. Granule cell membranes were added to Schwann cell cultures for 72 h at a concentration of 5.0–200.0 μgs of protein/ml. [^{3}H]Thymidine incorporation was determined by the cell harvester assay. Values are the average ± SD for $N = 4$.

Figure 3. Effect of heat, trypsin treatment, and NH$_4$Cl on granule cell membrane mitogenicity. Granule cell membranes were added to Schwann cells at a concentration of 50 μgs of protein/ml either untreated, added with 9 mM NH$_4$Cl, or after heat or trypsin treatment. The values are the fold change in [^{3}H]thymidine incorporation by Schwann cells over the amount of incorporation by non-treated controls ± SD for $N = 6$.
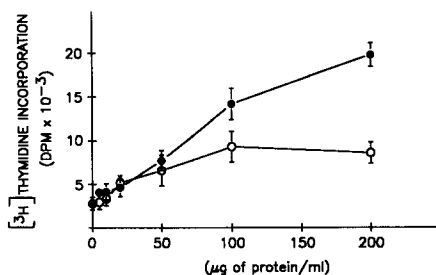
is found on astrocytes contaminating the granule cell cultures. Therefore, astrocytic membranes were isolated and added to Schwann cells (Fig. 2). Unlike granule cell membranes, astrocytic membrane had little effect on Schwann cell proliferation at doses above 50 μg/ml, the concentration at which granule cell mitogenicity increases.

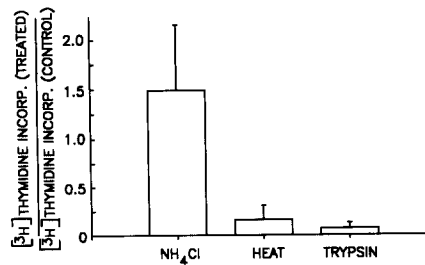## Effect of Heat, Trypsin, and NH$_4$Cl on the Granule Cell Mitogen

To determine the possible molecular nature of the granule cell mitogen, granule cell membranes were either heated or treated with trypsin before the addition of the membranes to the Schwann cell cultures. Heating of the granule cell membranes or trypsin treatment reduced the amount of [^{3}H]thymidine incorporation to 14 and 9% of nontreated membrane controls, respectively (Fig. 3). The sensitivity of the mitogen to heat and trypsin suggest that the mitogen may be a protein. NH$_4$Cl was added with granule cell membranes to Schwann cells to examine if the mitogenicity seen with the granule cell membranes was similar to the axolemma in its lack of dependency on lysosomal processing (31). Addition of NH$_4$Cl had no inhibitory effect on the granule cell membranes' induction of Schwann cell proliferation (Fig. 3).

Granule Cell and Schwann Cell Cocultures

To determine if the mitogenic effect seen for the granule cell membranes on Schwann cells is due to cell surface contact

and not due to a subcellular component found within the granule cell, Schwann cells were cocultured with intact granule cells (Fig. 4). Schwann cells cultured in the presence of granule cells (Table I) had a labeling index 30-fold higher than those cells cultured alone, which had a labeling index of 0.5%. This mitogenic signal appears to be due to cell contact since only Schwann cells found in contact with granule cells were labeled. Media from granule cell cultures was not mitogenic for quiescent Schwann cell cultures, suggesting that the mitogen is not a soluble factor (data not shown).

Effect of Proteoglycan Synthesis Inhibition on the Granule Cell Mitogen

To determine if the proliferative signal found on the granule cells is sensitive to the inhibition of proteoglycan synthesis, Schwann cells and granule cells were cocultured in the presence of the proteoglycan synthesis inhibitor 4-methylumbelliferyl-β-D-xyloside (Fig. 5). Schwann cells grown with granule cells in the presence of the inhibitor showed a 78% reduction in the number of labeled cells as compared to cells cocultured without the drug. The labeling index of the inhibitor-treated cultures was the same as seen for Schwann cells grown alone. Schwann cells cultured with granule cells in the presence of 4-methylumbelliferyl-α-L-arabinoside, a noninhibitory, structural analogue of β-D-xyloside, showed no decrease in the number of labeled Schwann cells.

Discussion

Development and maintenance of the nervous system depends on the proper communication between glial and neuronal cells. During development, glial cells have been implicated in the control of neuronal migration and axonal guidance (12, 23, 26). Cerebellar Bergman glia have been shown to control the migration of granule cells into the internal granule cell layer (8, 16, 17). Glial cells located in nerve grafts that had been placed adjacent to migratory granule cells in the external granule cell layer prevent the normal migration of the granule cells along the Bergman glia and cause the cells to migrate into the graft (11, 19, 20).

Neurons can also influence the development and differentiation of glial cells. Axons have been shown to influence glial

Figure 2. Astrocyte membrane effect on Schwann cell proliferation. Astrocyte membranes (*open circles*) and granule cell membranes (*closed circles*) were added to cultured Schwann cells at concentrations between 5 and 200 μg of protein/ml. [^{3}H]Thymidine incorporation was determined by the cell harvester assay. Values are the average ± SD for $N = 5$.
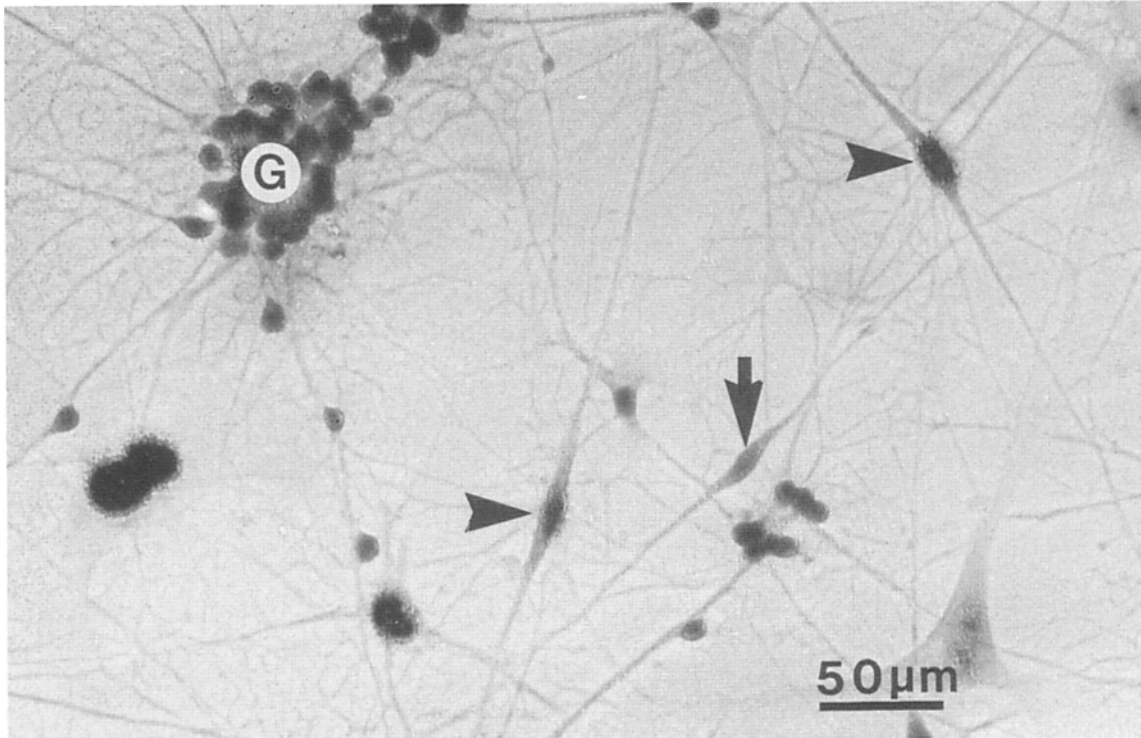
Figure 4. Coculture of Schwann cells and granule cells. Schwann cells were cultured with granule cells for 72 h at which time they were removed and processed for autoradiography. *Arrowheads,* labeled Schwann cells; *arrow,* unlabeled Schwann cell; *G,* granule cell bodies.

morphology (9), survival (5), ability to produce myelin (1, 27), and proliferation. Granule cells of the cerebellum have been shown to inhibit the rate of proliferation of both astrocytes (9) and astrocytoma cell lines (10). Axons have been shown to contain a signal that causes an increase in proliferation of Schwann cells both in vivo (1, 2, 25) and in vitro (29). The Schwann cell mitogen appears to be a polypeptide that may be linked to the membrane via a heparin sulfate proteoglycan. Little is known, however, about the mitogenic capacity of axons that will normally never encounter a myelin-competent glial cell. The granule cells of the cerebellum have been used in this study to determine if this Schwann cell mitogen is a ubiquitous factor found on axons of widely differing states of glial ensheathment, or whether it is located only on axons that will encounter a Schwann cell and ultimately be ensheathed or myelinated.

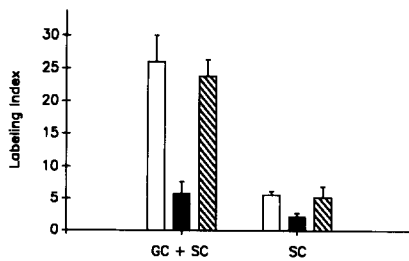Granule cell membranes, when added to cultured Schwann

Table I. Labeling Index of Schwann Cells Cocultured with Granule Cells

Time in culture	Labeling index for granule cell and Schwann cell cultures	Labeling index for Schwann cell controls	Fold stimulation
h			
48	18.5 ± 2.25	0.47 ± 0.45	39.2
72	15.3 ± 3.88	0.76 ± 0.65	20.2

Schwann cells were cocultured with granule cells for either 48 or 72 h with [³H]thymidine added for the last 24 h of the experiment and then processed for autoradiography. Values are the average labeling index ± SD for N = 3.

cells, produced a signoidally shaped dose–response curve. The Schwann cell mitogen found on the granule cell membrane is sensitive to both trypsin and heat treatment. The sigmoidally shaped dose–response curve and the sensitivity of the Schwann cell mitogen to heat and trypsin observed for granule cell membranes is similar to that found for axolemma-enriched fractions isolated from brain white matter (7, 31). The similarity of the granule cell membranes and the axolemmal membranes in their dose response and their sensitivity to heat and trypsin treatment suggest a similar Schwann cell mitogen may be present on both membranes. The Schwann cell mitogenic signal found on granule cells is sensitive to proteoglycan synthesis inhibition. This is similar to that reported for dorsal root ganglion neurons (18), suggesting that both mitogens might be associated with the membranes by a proteoglycan; again indicating that there is a common Schwann cell mitogen found on both types of neurons.

Salzer et al. (22) have shown that cell–cell contact is required for the neuronal mitogenic influence of Schwann cells. Meador-Woodruff et al. (14) have shown that axolemma-enriched fractions bind to the surface of the Schwann cells and are not internalized, suggesting that the mitogenicity seen for axolemma-enriched fractions might also be due to cell surface contact. The observed stimulation of Schwann cell proliferation by granule cells appears to be mediated through cell contact, because almost all of the labeled Schwann cells appear to be in contact with granule cell processes and the granule cell–conditioned media is not mitogenic to quiescent Schwann cell cultures. The mitogenic effect of granule cell membranes is not reduced by inhibition of the Schwann cell lysosomal processing, further suggesting that the proliferative signal acts through external stimulation.

Figure 5. Effect of proteoglycan synthesis inhibition on neuritic stimulation of Schwann cell proliferation. Schwann cells were incubated with granule cells for 48 h at which time either 1 mM of the proteoglycan synthesis inhibitor 4-methylumbelliferyl-β-D-xyloside (■) or 1 mM of the structural analog 4-methylumbelliferyl-α-L-arabinoside (▨) or media (□) was added for the last 96 h of the experiment. The values are the labeling index ± the average range for $N = 2$.

It is now apparent that the normally nonmyelinated, nonglial-ensheathed granule cells of the cerebellum have a mitogen for cultured Schwann cells. This proliferative signal appears similar to myelinated axolemma in its ability to stimulate cultured Schwann cells and its sensitivity to heat and trypsin treatment. The mitogen found on cultured granule cells appears similar to that found on dorsal root ganglion cultures in their ability to stimulate cultured Schwann cells and their sensitivity to inhibition of proteoglycan synthesis. Axolemma isolated from the splenic nerve of the autonomic nervous system, which has a Schwann cell ensheathment but is unmyelinated, is also mitogenic to cultured Schwann cells (Yoshino, J. E., and G. H. DeVries, unpublished observations). The findings of this paper suggest that the proliferative signal for Schwann cells might be a ubiquitous signal found on axons regardless of their ultimate state of glial ensheathment.

Since the Schwann cell proliferative response upon contacting an axon occurs independently of whether that Schwann cell will ensheathe or myelinate the axon, this suggests that the proliferative response may not be directly related to myelination. A signal found on an axon separate from the mitogenic signal may act alone or in concert with extracellular factors to control the glial cell production of a basal lamina and the formation of a myelin sheath. Therefore, we are currently investigating the ability of the granule cells to stimulate the production of basal lamina components or myelin-specific proteins in order to understand where the control lies for myelination. This information will ultimately help us understand the complex series of events that occur between the first contact of a glial cell and an axon and the formation of a compact myelin sheath.

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