Satellite Cell Proliferation in the Adult Rat Trigeminal Ganglion Results from the Release of a Mitogenic Protein from Explanted Sensory Neurons

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Abstract. Explant of trigeminal ganglia neurons in adult rats induces perineuronal glial proliferation of primarily satellite cells as opposed to Schwann cells. This proliferation begins at 15 h after explant culture and by 27 h there is a significant increase in glial proliferation as measured by scintillation counts of [^{3}H]thymidine. Blocking protein synthesis between 0 and 3.5 h after explant culture (early) results in an enhanced proliferative response, while blocking protein synthesis between 3.5 and 7 h (late) causes a complete block of the proliferative response assessed at 27 h. Conditioned media experiments demonstrate that both the mitogenic and inhibitory signals are diffusible and heat labile. Finally, the addition of neurotrophic factors to rescue injured ganglionic neurons attenuates the proliferative glial response suggesting that injured neurons produce and release signals that induce glial proliferation.

Axotomy induces morphological and biochemical changes in the neuronal cell body collectively known as the axon reaction (Lieberman, 1971, 1974; Grafstein and McQuarrie, 1978; Kreutzberg, 1982). The reaction of injured peripheral neurons can be classified into two general categories: regenerative or degenerative. The regenerative state is exemplified by the heightened tendency for neurons to grow after peripheral or central axotomy (McQuarrie et al., 1977; Richardson and Issa, 1984; Richardson and Verge, 1987). The degenerative state is typified by downregulation of certain proteins (Rotter et al., 1977; Tessler et al., 1985; Hoffman et al., 1987; Tetzlaff et al., 1988), neuronal atrophy and cell death (Aldskogius et al., 1978). Although the signals that trigger the various reactions in the cell body are not well understood, factors such as the type and proximity of the lesion affect the severity of the response (Lieberman, 1974). In sensory ganglia, these changes can be counteracted by the addition of exogenous growth factors (Otto et al., 1987; Rich et al., 1987) or upon reinnervation of the target (Rich et al., 1989).

Another response that accompanies the retrograde changes seen in axotomized neurons in both the central and peripheral nervous systems is the proliferation of perineuronal glial cells (Cammermeyer, 1965; Sjöstrand, 1965; Watson, 1965; Torvik and Skjörten, 1971). In the central nervous system, microglial cells proliferate after either axotomy or an injection of neurotoxin such as ricin (Pubols and Foglesong, 1988; Streit and Kreutzberg, 1988; Morshead and van der Kooy, 1990). Proliferating glial cells have the potential to phagocytize debris in the injured area (Pellegrino et al., 1986; Stoll et al., 1989) and to release growth factors that aid in the process of neuronal recovery (Politis and Miller, 1985; Lu et al., 1991). In autonomic or dorsal root ganglia, there are a number of instances when satellite glial cells proliferate: during development (Altman and Bayer, 1982; Lawson et al., 1974; Hall and Landis, 1992), during injury (Humberston et al., 1969, Pubols and Foglesong, 1988; Lu and Richardson, 1991), in pathological states (Hanker, 1976; for review see Prineas and Spencer, 1975), after repeated stress (Dropp and Sodetz, 1971), and after electrical stimulation of preganglionic nerve fibers (Schwyn, 1967). Identification of the signals which trigger glial proliferation after injury may shed light on the events which lead to the induction of satellite cell proliferation under all the above conditions.

An important neuronal–glial signaling mechanism that has not been clearly elucidated is the induction of glial proliferation after neuronal injury. We are studying a sub-population of satellite cells that proliferate and are not labeled with glial markers (Glial fibrillary acidic protein, Vimentin, and S-100) when neurons of the trigeminal ganglion are explanted.[1] To gain insight into the signaling mechanisms between injured neurons and their resident glial cells, an explant culture system was used. This explant system has the advantage of eliminating the influence of blood cells that are normally present during the injury process (Perry et al., 1987; Stoll

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et al., 1989). Blood cells invading the injury site in vivo contaminate the analysis of the reaction of the endogenous population of glial cells in response to injury. Explant cultures also have the advantage of preserving the in vivo structure of the ganglion. It is likely that the maximum response to injury will be seen after both peripheral and central branch axotomy (as in explant cultures); axotomizing either branch alone produces partial effects (Lieberman, 1974; Perry et al., 1983). The major goal of this study was to ask about the nature of the signal, when and where the signal for injury-induced glial proliferation is produced. Second, we sought to determine whether neurotrophins such as NGF or brain-derived neurotrophic factor (BDNF)[2] inhibit explant-induced glial proliferation.

Materials and Methods

Surgery

Adult male Charles River rats (250–300 g) were anesthetized with a 0.2 ml injection (i.p.) of Sodium Pentobarbitol (65 mg/kg), and then decapitated. The trigeminal ganglion on both sides of the head was removed (under sterile conditions) by first peeling off the overlying dura mater and then cutting the ophthalmic, maxillary, and the mandibular peripheral branches as they leave the skull. The central branch of the trigeminal ganglion was cut at a point parallel to the posterior aspect of the pituitary gland. After removing the ganglia, they were immediately placed in preheated serum-free medium (described below). The tissue capsule and the surrounding blood vessels were then carefully and quickly removed under a dissecting microscope. The ganglia were then placed in their respective sterile petri dishes (two ganglia per dish) containing 3 ml of serum-free media in an incubator set at 37°C and 5.0% CO_2.

Tissue Culture

Ganglia were cultured in serum-free medium which contained 80% DME (GIBCO BRL, Gaithersburg, MD), 20% Ham's F-12 (Sigma Chemical Co., St. Louis, MO) and [^{3}H]thymidine (47 Ci/mmol; Amersham Corp., Arlington Heights, IL) which was diluted down to a final concentration of 2 μCi/ml. An insulin-transferrin-sodium selenite medium supplement (Sigma Chemical Co.) and a Penicillin, Streptomycin, and Neomycin antibiotic mixture (GIBCO BRL) were added to the serum-free medium and diluted to 1% before use. Cycloheximide cultures contained 1 μg/ml of cycloheximide (Sigma Chemical Co.). This was diluted from a 10 mg/ml stock solution of cycloheximide dissolved in 100% ethanol. To remove cycloheximide from cultures, ganglia were put through three 5-min washes, each consisting of placing exposed ganglia to preheated serum-free medium while mixing intermittently. NGF (50 ng/ml) (Sigma Chemical Co.) and BDNF (50 ng/ml) (Regeneron Pharmaceuticals Inc., Tarrytown, NY) were prediluted in serum-free medium before the addition of two ganglia to each petri dish.

Conditioned Media

Conditioned medium was prepared by placing two axotomized ganglia in 3 ml of serum-free media at the proper incubation conditions. Collection of medium which contained the inhibitory signals was accomplished by allowing two explanted ganglia to incubate for the first 3.5 h in culture. After this time, they were removed and immediately replaced with two freshly explanted ganglia. These ganglia were left for an additional 27 h in the presence of [^{3}H]thymidine before the tissue was processed.

Conditioned medium containing the mitogenic signal was obtained by placing two ganglia in 3 ml of serum free medium for a total of 27 h. After this time, they were removed and replaced with two freshly explanted ganglia. Another 27 h of incubation in the presence of [^{3}H]thymidine was allowed to elapse before the ganglia were processed. The effects of 0–48 h conditioned medium (collected between 0–48 h after injury) on [^{3}H]thymidine incorporation was also assessed. In additional experiments, a single ganglion was cultured in conditioned medium collected from four ganglia or one ganglion preparation to look for an effect of concentration.

2. *Abbreviations used in this paper*: BDNF, brain-derived neurotrophic factor; GGF, glial growth factor.

Heating Conditioned Media

Conditioned medium containing both the inhibitory and the mitogenic signals were collected as stated above and subjected to heat treatment. The two types of conditioned medium were placed in different test tubes and heated at 65°C in a hot water bath for a period of 1 h. After cooling the media back to 37°C, two freshly explanted ganglia per petri dish were placed in the heat-treated media. The ganglia in these cultures were left to incubate for 27 h in the presence of [^{3}H]thymidine.

Tissue Preparation and Scintillation Counting

Ganglia were removed from the tissue culture medium after the appropriate incubation time and placed in ependorff tubes. They were stored at −20°C before tissue processing. Each ganglion was thawed and its wet weight recorded. After mechanical dissociation with tweezers, each ganglion was placed in a 10-ml conical test tube containing 1 ml of an enzyme solution containing 0.25% trypsin, and 0.125% collagenase (Sigma Chemical Co.) in 0.1 M PBS, pH 7.2. The test tubes were stored in an incubator overnight at 37°C for enzymatic dissociation. The test tubes were then vortexed at high speeds until all the contents within the tube were pulverized and suspended. A 1% SDS solution was added to the tubes until a final volume of 4 ml was reached. The mixture was then mixed thoroughly using a vortex. All the tubes were spun down in a centrifuge at 100 *g* for 1 min to pellet any remaining particles that were not dissolved. Duplicate samples for each time point were prepared by pippeting 30-μl samples onto 1.5-cm glass micro filter paper (Ahlstrom Filtration Inc., Mt. Holly Spring, PA). After drying, the samples were put through a TCA precipitation by running samples through a series of solutions (10 min each) in the following order: 10% TCA, 5% TCA (both dissolved in 0.1 M PBS), 95% ethanol, and 95% ethanol. The samples were then dried completely before putting them into glass vials containing 10 ml of scintillation fluid (Aquasol Universal LSC Cocktail for Aqueous Samples; NEN Research Products, Boston, MA). The vials sat for 1 h before they were counted for [^{3}H]thymidine on a Beckmann LS 1800 scintillation counter (Beckman Instr. Inc., Fullerton, CA). Background radiation counts were obtained by placing unprocessed microglass filters into 10 ml of scintillation fluid and counted with experimental samples.

BrdU and [^{3}H]thymidine Double Labeling

Explanted ganglia were placed in culture containing 3 μg/ml of bromodeoxyuridine (BrdU) (Sigma Chemical Co.) for the first 34 h after injury. The same ganglia were then immediately transferred to a culture containing [^{3}H]thymidine diluted to 2 μCi/ml. These ganglia were then allowed to incubate for another 14 h before the ganglia were removed at 48 h, frozen, and processed for both BrdU and [^{3}H]thymidine labeling.

[^{3}H]thymidine Autoradiography and BrdU Processing

Ganglia were cultured in serum-free culture medium containing either [^{3}H]thymidine or BrdU and then placed in a solution containing 20% sucrose in 10% formalin for 24 h. Sections (5 μm) were cut on a cryostat at −20°C and mounted on microscope slides coated with 3-aminopropyltriethoxysilane (Sigma Chemical Co.). Before BrdU processing, sections were washed in 3% H_2O_2 in methanol for 10 min and then rinsed in 100% ethanol to remove endogenous peroxidases. These sections were incubated in 0.7 N NaOH for 10 min and washed in 0.1 M PBS for 30 s. Sections were then incubated for 1 h in a primary monoclonal antibody 1:50 (diluted in normal horse serum) directed against single stranded DNA containing BrdU (Becton-Dickinson, Mountain View, CA). After three 5-min washes in 0.1 M PBS, sections were incubated in a biotinylated horse anti–mouse IgG (Cappel Laboratories, Malvern, PA) at 1:50 for 1 h. After another three 5-min washes, sections were incubated with streptavidine-horseradish peroxidase conjugate (GIBCO BRL) at 1:62.5 for an additional hour. After a final three 5-min washes, sections were soaked in a solution of DAB (Sigma Chemical Co.) at a concentration of 1 mg/ml. Sections were allowed to sit in this solution for ~1.5 min after the addition of 0.017% of H_2O_2. After BrdU processing, sections were defatted in xylene and dehydrated in increasing concentrations of alcohol. Sections were processed for [^{3}H]thymidine autoradiography by dipping the slides in liquid emulsion (NTB-2) and allowing 5 wk of exposure in the dark. Finally, the sections were counterstained with Nissl and coverslipped with glycerol:water (10:1).

Quantification of Cell Counts

Sections from four ganglia containing all three trigeminal cell body regions

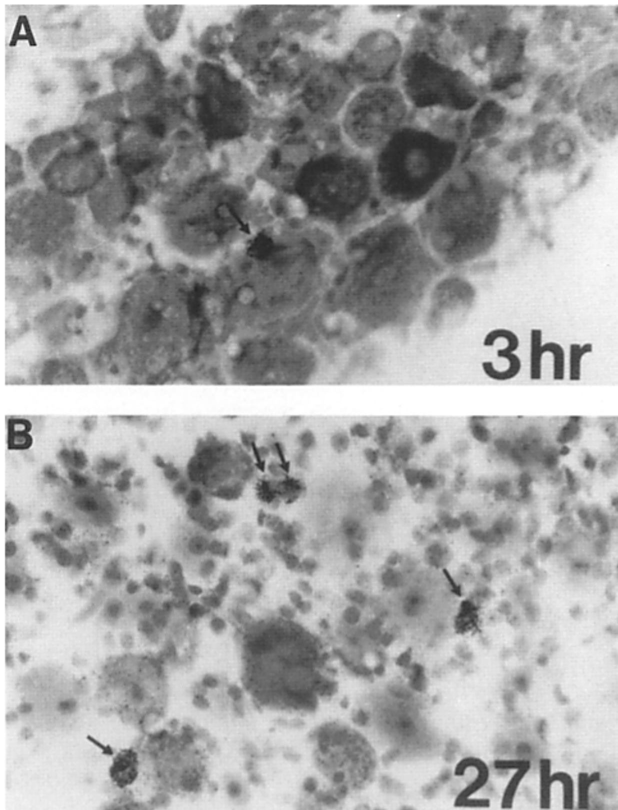

Figure 1. Autoradiographs of adult trigeminal ganglion sections 3 h (*A*) and 27 h (*B*) after injury and explant culture. Arrows show satellite cells that have incorporated [^{3}H]thymidine. There are increased numbers of satellite cells labeled at 27 h after injury.

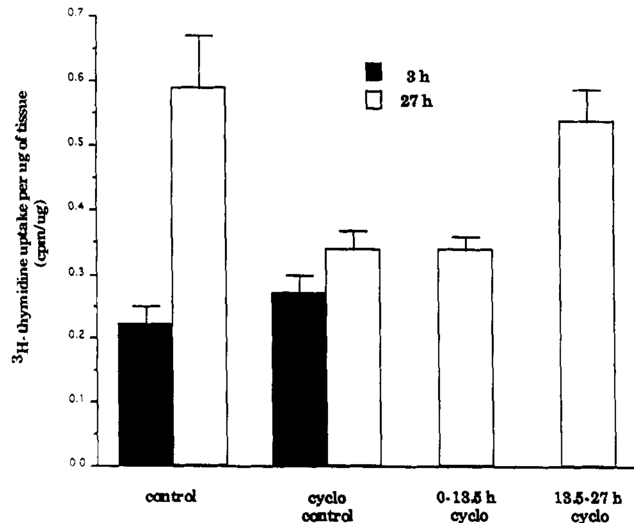

Figure 2. [^{3}H]thymidine incorporation in trigeminal ganglia cultured in the presence and absence of cycloheximide (1 μg/ml) for various periods. Control ganglia (*control*) were cultured for 3 (n = 7 ganglia) and 27 h (n = 8) after injury. In the cycloheximide control condition (*cyclo control*), ganglia were incubated in the presence of continuous cycloheximide for 3 (n = 8) and 27 (n = 8) h after injury. In the 0–13.5-h cycloheximide condition (*0–13.5 cyclo*) (n = 8), ganglia were exposed to cycloheximide for the first 13.5 h of the 27-h explant culture period. After the ganglia were rinsed, they were placed back into a culture in the absence of cycloheximide. In the 13.5–27 h cycloheximide condition (13.5–27 h cyclo) (n = 8), ganglia were exposed to cycloheximide from 13.5 h after injury until the end of the 27 h culture period. All cultures contained [^{3}H]thymidine continuously. Bars represent means ± SEMs.

were selected for counting. The number of sections counted for each of the four ganglia were 12, 14, 16, and 12, respectively. Every glial cell labeled with a proliferation marker (both in the cell body and the fiber tract region) was counted on each section. Labeled cells were separated on the basis of their location: either in the cell body or fiber tract regions. Only cells within three glial cell diameters of a neuronal cell body were considered to be satellite cells, while the other glial cells were scored as fiber tract cells. Within each region, cells were scored as possessing either [^{3}H]thymidine, BrdU, or both proliferation markers. [^{3}H]thymidine-positive glial cells were scored if grain counts were five times greater than above a similar area of background tissue, while BrdU-positive glial cells were easily scored by their dark DAB staining compared with the background staining of the tissue. The data represent labeled cells as a percentage of the total cells within either the cell body or the fiber tract regions. Data for each cell type are expressed as average percentages per section of labeled cells out of the total number of glial cells in either the cell body or fiber tract regions. The total number of glial cells per section from each ganglion was obtained by counting all the glial cells in representative sections from each ganglion.

Results

Satellite Cells in Explanted Trigeminal Ganglia Show Both a Morphological Change and a Proliferative Response

Injury was induced by explant culture of the adult trigeminal ganglion. After 27 h in explant culture, two injury induced changes are noticeable: a morphological change and an injury-induced glial cell proliferation. At 0 or 3 h after injury, perineuronal satellite cells appear as flattened, encapsulating cells with a very low proliferation rate (Fig. 1 *A*). By 27 h after injury, satellite cells moved away from the neuronal cell bodies, took on a more spherical appearance, and a larger number showed [^{3}H]thymidine labeling than at 3 h after injury (Fig. 1 *B*). Neuronal cell bodies were never labeled with [^{3}H]thymidine in the trigeminal explant cultures. A very low baseline [^{3}H]thymidine incorporation proliferative response, not significantly different from background scintillation counts ($t(6) = 1.4$; $P > 0.05$) was seen at 3 h after injury. However, by 27 h there was a significant increase ($t(10) = 5.1$; $P < 0.05$) from 0.38 ± .02 cpm/μg of tissue at 3 h to 1.58 ± 0.17 cpm/μg of tissue at 27 h. This increase in [^{3}H]thymidine incorporation has been shown to start at 15 h after injury.[1] However, 27 h is not the peak response, since extending culture times to 48 h produces a significant increase over 27 h in the amount of [^{3}H]thymidine incorporation to 7.85 ± 0.41 cpm/μg of tissue ($t(10) = 18.6$; $P < 0.05$).

Ganglia Incubated in the Presence of Cycloheximide for 27 h Show Complete Suppression of Proliferation

To determine whether protein synthesis was necessary for the induction of glial proliferation, cycloheximide (1 μg/ml) was added to cultures for the entire culture time (either 3 or 27 h). Little glial proliferation was seen in either the presence or absence of cycloheximide at 3 h after injury. However, in the presence of cycloheximide, glial proliferation at 27 h after injury was totally suppressed compared with the

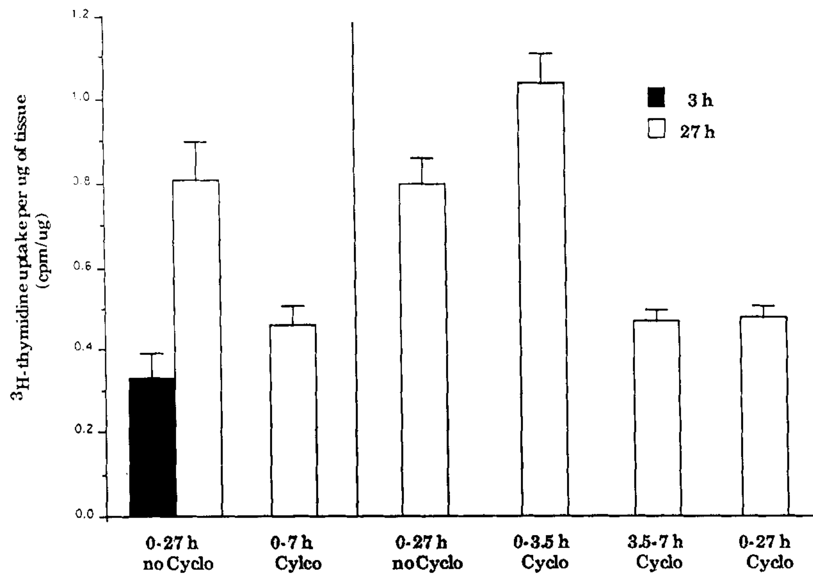

Figure 3. (*Left*) The cycloheximide-sensitive window can be further narrowed to the first 7 h after injury. Control ganglia (*control*) were cultured in the absence of cycloheximide for 3 and 27 h. The 0–7 cyclo group ganglia were exposed to cycloheximide for only the first 7 h of the 27-h explant culture period. (n = 4, 8, and 8 ganglia from left to right in the three groups on the left of the graph). (*Right*) Different temporal exposures of cycloheximide produce opposite effects on proliferation. The 0–27 h no cyclo (n = 12) and 0–27 cyclo (n = 12) ganglia were cultured for 27 h in the absence and in the presence of cycloheximide, respectively. In the 0–3.5 h cyclo condition (n = 16), ganglia were cultured in the presence of cycloheximide for only the first 3.5 h of the 27-h explant culture period, while in the 3.5–7-h cyclo condition (n = 12) ganglia were exposed to cycloheximide only during the 3.5–7-h period after injury and then rinsed before the continued culture until 27 h after injury. All cultures contained [^{3}H]thymidine continuously. Bars represent means ± SEMs.

proliferation seen in the absence of cycloheximide after 27 h (Fig. 2). A two-way ANOVA on the [^{3}H]thymidine incorporation values revealed a significant interaction between culture time (3 or 27 h) and the absence or presence of cycloheximide ($F_{1,27} = 19.4$, $P < 0.05$). Given that cycloheximide acts on both neurons and glia in explant cultures, it is possible that rather than blocking synthesis of an injury signal in neurons, the cycloheximide is blocking the synthesis of proteins in glial cells necessary for DNA synthesis. In an attempt to differentiate between these possibilities, cycloheximide exposure was limited to either the first or the last 13.5 h of the 27-h explant culture period. A time course study[1] has revealed that DNA synthesis in glial cells does not begin until at least 15 h into the 27-h explant culture period. This finding allows us to ask whether glial cells can undergo DNA synthesis in the presence of cycloheximide during the last 13.5 h of the 27-h culture period. The results show that once satellite cells are induced to proliferate in the first 13.5 h, they can take up [^{3}H]thymidine in the presence of cycloheximide in the last 13.5 h of the culture period (Fig. 2). The [^{3}H]thymidine incorporation seen in cultures in which the application of cycloheximide was restricted to the latter 13.5-h period is not significantly different from that in untreated 27 h control cultures ($t(14) = 1.0$; $P < 0.05$). However, limiting cycloheximide to the first 13.5 h produced the same effects on proliferation as continuous cycloheximide cultures; total suppression of [^{3}H]thymidine uptake compared with untreated 27-h control explant cultures ($t(14) = 4.2$; $P < 0.05$).

Blocking Protein Synthesis Reveals Two Components of the Proliferative Response within the First 7 h of Injury

The previous experiment revealed that blocking protein synthesis from 0–13.5 h in culture totally suppressed the proliferative response seen at 27-h in explant culture. This protein synthesis-sensitive window can be narrowed further. Cycloheximide (cyclo) exposure for 0–7 h of the 27-h explant culture period completely blocked the injury-induced glial proliferative response. The 0–7 h cyclo groups showed significantly less ($t(14) = 3.8$; $P < 0.05$) [^{3}H]thymidine incorporation after 27 h compared with a 27-h control group. Indeed, incorporation by the 0–7 h cyclo group was not significantly different from 3-h controls ($t(10) = 1.5$; $P > 0.05$) (Fig. 3, *left*). Exploring this 7-h period further revealed the existence of two windows in which blocking protein synthesis produces opposite effects on glial proliferation. Blocking protein synthesis in the first 3.5 h after explant culture (0–3.5 h cyclo) resulted in a significantly enhanced proliferative response at 27 h compared with control ganglia cultured in the absence of cycloheximide for 27 h (0–27 h no cyclo) ($t(26) = 2.6$; $P < 0.05$) (Fig. 3, *right*). However, blocking protein synthesis only between 3.5 and 7 h produced a suppressed proliferative response when assessed at 27 h compared with control ganglia cultured in the absence of cycloheximide for 27 h ($t(22) = 4.7$; $P < 0.05$). The inhibition of proliferation in the 3.5–7-h cyclo group was not significantly different from the inhibition seen after continuous cycloheximide exposure for the same 27-h period in the 0–27 h cyclo group ($t(22) = 0.7$; $P > 0.05$).

Conditioned Medium Reveals the Diffusible Nature of the Signals

Conditioned medium experiments were designed to investigate whether the inhibitory and mitogenic glial proliferation signals are diffusible or membrane bound. Conditioned medium collected at appropriate times reproduced the mitogenic and inhibitory effects on glial proliferation (Fig. 4) as predicted from the cycloheximide experiments. The effects of conditioned medium collected from cultures that were exposed for the first 3.5 h or for the full 27 h after injury were compared with unconditioned medium. A one-way ANOVA revealed a significant effect of the type of media used during culturing on [^{3}H]thymidine incorporation ($F_{2,41} = 52.6$, $P <$

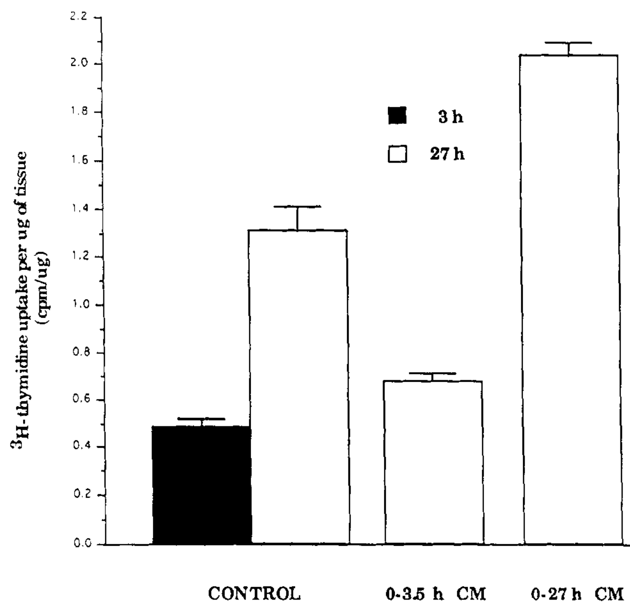

Figure 4. Effects of conditioned media on glial cell proliferation. Control ganglia were cultured for 3 (n = 8) and 27 h (n = 20) after injury. In the 0–3.5-h CM condition (n = 12), ganglia were cultured for 27 h in conditioned media collected between 0 and 3.5 h after injury. In the 0–27-h CM condition (n = 10), ganglia were cultured for 27 h in conditioned media collected between 0 and 27 h after injury. All cultures contained [^{3}H]thymidine. Bars represent means ± SEMs.

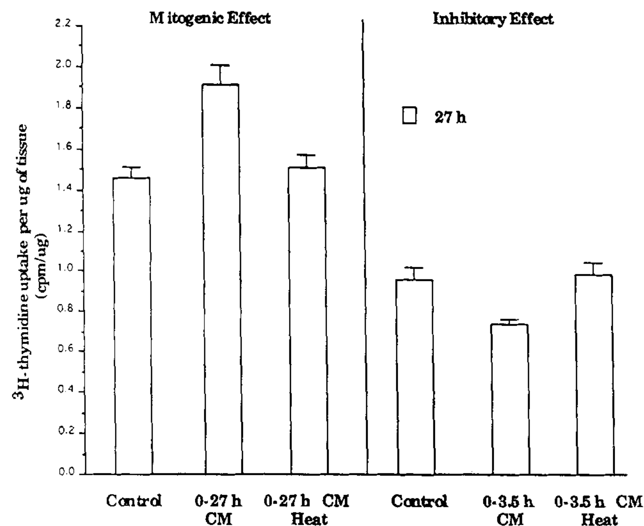

Figure 5. Both mitogenic and inhibitory signals on proliferation are heat labile. The inhibitory (collected between 0 and 3.5 h) and the mitogenic media (collected between 0 and 27 h) were heated at 65°C for 1 h before culturing. Bars represent ± SEMs (n = 4, 4, 4, 8, 12, and 12 ganglia, from left to right).

0.05). Ganglia cultured for 27 h in conditioned medium collected between 0 and 27 h after injury showed a significant increase in [^{3}H]thymidine incorporation when compared with a 0–27-h unconditioned control group (Newman Keuls; $P < 0.05$). However, ganglia cultured for 27 h in conditioned medium collected between 0 and 3.5 h after injury showed a significant suppression of [^{3}H]thymidine incorporation when compared with a 0–27-h unconditioned control group ($P < 0.05$). This suppressed proliferation was still significantly higher than a control group cultured in unconditioned medium for 3 h ($t(22) = 2.8$; $P < 0.05$). The 0–27-h conditioned medium may also contain the inhibitory factor, but the net effect that is seen is enhanced glial proliferation. This suggests that the mitogenic factor is somehow overcoming the suppressive effects of the inhibitory factor.

To ask if the increased induction of glial proliferation seen after 48 h compared with 27 h in culture was simply a response to a higher and growing concentration of the same mitogen acting at 27 h, several types of conditioned media were compared. First, we assessed the effects of conditioned medium collected between 0 and 27 h from one versus four ganglia on [^{3}H]thymidine incorporation. Ganglia cultured for 27 h in conditioned medium collected from four ganglia did not show significantly greater [^{3}H]thymidine incorporation than ganglia cultured in conditioned medium collected from one ganglion ($t(6) = 1.0$; $P > 0.05$). We assume that these conditioned media effects on glial proliferation are supramaximal, although experiments to dilute out the effects were not done. Second, we compared ganglia cultured in conditioned media collected between 0–48 h and 0–27 h with ganglia cultured for 27 and 48 h in unconditioned media. In addition to testing the mitogen concentration idea, this experiment also speaks to the idea of a putative second diffusible glial mitogen that may be responsible for the significant increase in [^{3}H]thymidine incorporation seen at 48 h. As seen previously, ganglia cultured in conditioned medium collected between 0 and 27 h showed a significant increase in the amount of [^{3}H]thymidine incorporation when compared with the 27-h unconditioned control group ($t(6) = 3.3$; $P < 0.05$). However, there were no statistically significant differences between the 0–48-h conditioned group and the 0–27-h unconditioned control group ($t(6) = 0.6$; $P > 0.05$). These results suggest that the increase in glial proliferation seen after 48 h in culture does not depend on increasing concentrations of the same mitogen or another diffusible glial mitogen.

Heating Conditioned Medium Abolishes Their Effects on Proliferation

The conditioned medium experiments showed that both the mitogenic and inhibitory signals are diffusible. To ask if both of these signals are proteins, conditioned media collected between 0–3.5 and 0–27 h after injury were heated at 65°C for 1 h before experimental culturing. Heating destroyed both the mitogenic and inhibitory signals, suggesting the diffusible factors are proteins. First, the mitogenic signal was assessed by investigating the effects of three different types of media (unconditioned control, 0–27 h conditioned and heat-treated 0–27 h conditioned) on [^{3}H]thymidine incorporation (Fig. 5, *left*). A one-way ANOVA revealed a significant effect of the type of media used ($F_{2,11} = 15.8$, $P < 0.05$). Ganglia cultured in 0–27-h conditioned medium showed significantly more [^{3}H]thymidine incorporation than the 0–27-h unconditioned control group (Newman Keuls; $P < 0.05$). However, ganglia cultured in heat-treated conditioned medium showed a significant reduction in [^{3}H]thymidine incorporation com-

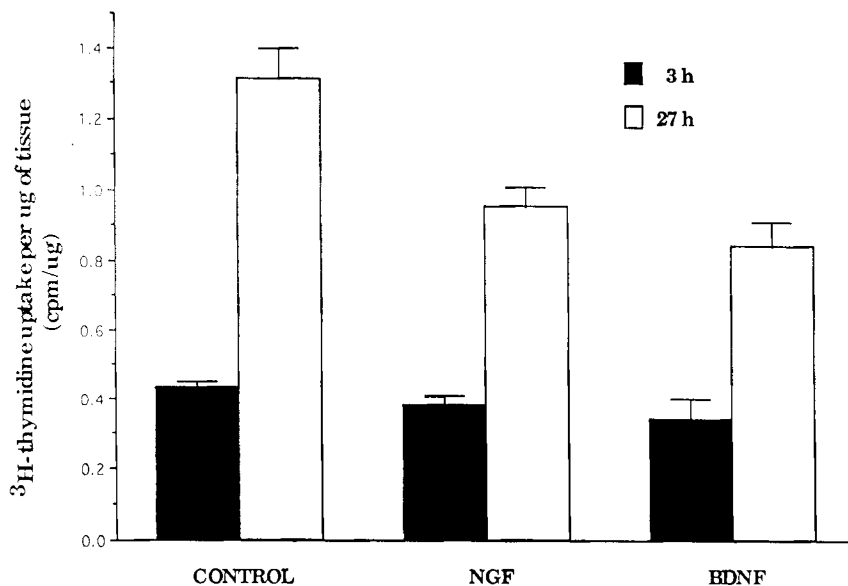

Figure 6. Effects of trophic factors on injury induced glial proliferation in axotomized trigeminal ganglia. Control ganglia were cultured for 3 (n = 12) and 27 h (n = 12) after injury. In NGF cultures, ganglia were exposed cultures containing 50 ng/ml of NGF for 3 (n = 12) and 27 h (n = 12). In BDNF cultures, ganglia were exposed to 50 ng/ml of BDNF for 3 (n = 3) or 27 h (n = 4). All cultures contained [^{3}H]thymidine continuously. Bars represent means ± SEMs.

pared with the 0–27-h conditioned medium group ($P < 0.05$). Indeed, this reduced incorporation of [^{3}H]thymidine was not significantly different from that observed in the 0–27-h unconditioned control group ($P > 0.05$). Thus, the mitogenic effect of 0–27-h conditioned medium was totally abolished after heat treatment.

Second, the inhibitory signal was similarly investigated by heating conditioned medium collected between 0 and 3.5 h after injury before experimental culturing for 27 h (Fig. 5, *right*). A one-way ANOVA revealed a significant effect of media type (unconditioned control, 0–3.5-h conditioned and heat-treated 0–3.5-h conditioned) on [^{3}H]thymidine incorporation ($F_{2,27} = 8.56$, $P < 0.05$). Ganglia cultured in conditioned medium collected between 0 and 3.5 h after injury showed significantly less [^{3}H]thymidine incorporation compared with a 0–27-h unconditioned medium control group (Newman Keuls; $P < 0.05$). Heat treatment of this conditioned medium significantly increased the [^{3}H]thymidine incorporation by ganglia compared with those cultured for 27 h in 0–3.5-h conditioned medium ($P < 0.05$). Moreover, this heat induced increase in [^{3}H]thymidine incorporation was not significantly different from that seen in the 0–27-h control group ($P > 0.05$).

Exogenous Neurotrophins Attenuate the Proliferative Response

The origin of the signal causing glial proliferation is unknown. However, given that the initial glial proliferation is anatomically localized around neuronal cell bodies at 27 h in vitro, it is reasonable to hypothesize that injured neurons provide the signal for glial proliferation. To test this hypothesis, trophic support for the neurons in explant culture was provided by adding exogenous neurotrophins (NGF and BDNF). After ganglia incubation times of 3 or 27 h in the presence of either growth factor, scintillation counts were obtained and compared with those obtained from control ganglia cultured in the absence of growth factor for the same amount of time. Dose-response curves using 25, 50, 100, and 500 ng/ml of either NGF or BDNF showed that the greatest

Table I. The Numbers of Proliferating Glial Cells Expressed as Mean Percentages per Section of All Glial Cells at 48 h in Culture

	Cell body			Fiber tract			Number of counted sections
	[^{3}H]thymidine	BrdU	[^{3}H]thymidine + BrdU	[^{3}H]thymidine	BrdU	[^{3}H]thymidine + BrdU	
Ganglia 1	0.026 ± 0.0002	0.0028 ± 0.00037	0.0047 ± 0.00092	0.0026 ± 0.00029	0.00046 ± 0.000062	0.00055 ± 0.00018	12
Ganglia 2	0.034 ± 0.0095	0.0012 ± 0.00044	0.0100 ± 0.00320	0.0042 ± 0.00067	0.00086 ± 0.000220	0.00150 ± 0.00028	14
Ganglia 3	0.019 ± 0.0026	0.0052 ± 0.00099	0.0140 ± 0.00230	0.0017 ± 0.00034	0.00093 ± 0.000220	0.00320 ± 0.00019	12
Ganglia 4	0.029 ± 0.0021	0.0034 ± 0.00053	0.0056 ± 0.00110	0.0036 ± 0.00062	0.00059 ± 0.000095	0.00069 ± 0.00014	16
Means	0.027 ± 0.0036	0.0032 ± 0.00095	0.0086 ± 0.00250	0.0030 ± 0.00064	0.00071 ± 0.000130	0.00150 ± 0.00070	54

Values represent the number of labeled glial cells expressed as mean percentages per section of all of the Nissl-stained glial cells found either in the cell body or fiber tract region after 48 h in explant culture. Only sections (5 μm) which contained all three cell body regions were counted. Trigeminal ganglia were incubated in cultures containing BrdU (early proliferation marker) for the first 34 h, and then transferred to cultures containing [^{3}H]thymidine (late proliferation marker) until 48 h after injury. Double-labeled cells ([^{3}H]thymidine and BrdU) represent single glial cells that proliferate twice (both early and late in the 48-h culture period). Values represent mean percentages ± SEMs.

suppressive effects of either growth factor on [^{3}H]thymidine incorporation over 27 h in culture were seen with 50 ng/ml (data not shown). Both NGF and BDNF reduced the glial proliferative response seen in the 27-h cultures (Fig. 6). A two-way ANOVA of [^{3}H]thymidine incorporation revealed a significant interaction of culturing for 3 h versus 27 h with the absence or presence of growth factors ($F_{2,49} = 5.5$, $P < 0.05$). At 3 h after injury, minimal glial proliferation was seen in the presence of either NGF or BDNF or in their absence. However, a reduction of [^{3}H]thymidine incorporation at 27 h after explant culture was seen with the addition of either NGF or BDNF (Newman Keuls; $P < 0.05$) compared with the 27 h control cultures. Although [^{3}H]thymidine incorporation after 27 h in BDNF was slightly less in NGF, this difference did not reach statistical significance ($P > 0.05$). We believe that NGF is acting specifically on neurons (and not satellite cells) since only a sub-population of neurons in the trigeminal ganglion and not satellite cells express the *trk* A receptor (Verge et al., 1992). However, it is not clear at this point whether in addition to neuronal expression, some satellite cells in the ganglion also express *trk* B mRNA. (Verge, V. M. K., personal communication). These results demonstrate that providing trophic support to axotomized neurons can reduce the glial proliferative response.

Double Labeling Reveals Three Different Sub-populations of Proliferating Satellite Cells

We examined whether the increased proliferation seen after 48 h in culture compared with shorter culture times represented the continued division of one population of cells or the division of additional populations. Ganglia were initially incubated in a culture containing BrdU for the first 34 h after injury and then transferred to a culture containing [^{3}H]thymidine until 48 h. The data shows that there are at least three populations of satellite cells that can be distinguished (Table I): an early labeled population that entered S phase once (BrdU only), a population that entered S phase twice (double labeled), and a relatively large population that entered S phase after 34 h in culture and hence only possess the late proliferation marker ([^{3}H]thymidine only). The majority of proliferating cells are localized around neuronal cell body as opposed to fiber tract regions, as revealed by the significant main effect of location ($F_{1,18} = 71.0$, $P < 0.05$) in the two-way ANOVA comparing the location and types of labeled cells. The predominant type of labeled cell seen possesses only the late proliferation marker ([^{3}H]thymidine) as revealed by the main effect of labeled cell types ($F_{2,18} = 35.5$, $P < 0.05$). Thus the largest number of labeled cells are those cells that incorporated [^{3}H]thymidine only after 34 h after injury. There was also a significant interaction between the types of labeled cells and the region they are found in ($F_{2,18} = 24.4$, $P < 0.05$). This interaction reflects the especially large increase in the number of [^{3}H]thymidine labeled cells found in the cell body region compared with the fiber tract region. This result suggests a particularly large population of satellite cells proliferating in the neuronal cell body region only during the latest stage of the 48-h culture period. It should also be noted that the majority of proliferating fiber tract cells are restricted to the regions near the cut stumps of the trigeminal ganglion.

Discussion

Injury of the trigeminal ganglion causes the proliferation of satellite glial cells. The proliferation kinetics of these cells shows that glial cells do not enter S phase for at least 15 h after explant culture.[1] This lag period may indicate the time needed for the production and the release of proteins mitogenic for satellite glial cells. To test this idea, the protein synthesis inhibitor cycloheximide was used to block the production of any putative mitogen, and hence block the glial response. The present experiments revealed that blocking protein synthesis continuously for 27 h in culture completely eliminates the glial response due to explant culture. There are, however, several interpretations of this data. First, it is possible that blocking neuronal protein synthesis blocks the production of a mitogen for satellite glial cells and thus suppresses any glial response. Second, it is also possible that the addition of cycloheximide prevented injured neurons from cell death (Martin et al., 1988) and therefore prevented satellite glial cell proliferation. Finally, because both neuronal and glial protein synthesis are blocked, the resulting suppression of glial proliferation may be due to a direct block of glial protein synthesis. The importance of protein synthesis for the production of cyclins and the importance of cyclins for progression through the cell cycle are well documented (Girard et al., 1991; Nurse, 1990; Swenson et al., 1989). Limiting cycloheximide to the first 13.5 h in culture (a period when no glial proliferation occurs) completely suppressed the incorporation of [^{3}H]thymidine when assessed 27 h after explant culture. However, adding cycloheximide to cultures between 13.5 and 27 h does not significantly suppress proliferation when assessed at 27 h, suggesting that glial cells can incorporate [^{3}H]thymidine during S phase in the presence of cycloheximide once they have been induced to re-enter the cell cycle. Taken together, these results indicate that protein synthesis is important in the first 13.5 h of the culture period for the induction of the glial response, possibly for the neuronal synthesis of a mitogen for glial satellite cells.

Further investigation of this 0–13.5-h time period revealed two components of the proliferative response. Blocking protein synthesis between 0 and 3.5 h resulted in an enhanced proliferative response while blocking protein synthesis between 3.5 and 7 h suppressed the proliferation. We suggest that blocking protein synthesis early blocks the production of an inhibitory signal while blocking protein synthesis late may block the production of a mitogenic signal. The fact that opposite effects on glial proliferation can be seen in response to the same total duration of cycloheximide exposure (3.5 h) argues against suppressive effects of any residual cycloheximide left in culture after washing.

In principle, there are two possible intercellular signaling mechanisms that can activate glial proliferation: a contact-mediated versus a diffusible mechanism. Satellite cells in uninjured ganglia are in close apposition to neuronal cell bodies, providing the basis for a cell contact-mediated signaling mechanism. In the injured ganglia, satellite cells proliferate and seem to move away from neuronal cell bodies. It is possible that the loss of neuronal contact could induce glial proliferation, although contact-mediated mechanisms have been implicated in the control of Schwann cell proliferation (Eccleston et al., 1989) and differentiation (Morgen et al., 1991) during development. However, it is

also possible that diffusible signals are responsible for the induction of the glial mitogenic and inhibitory responses. Conditioned media experiments were performed to investigate the nature of the signals. The results of these experiments revealed that conditioned media collected at appropriate times can mimic the inhibitory and mitogenic responses obtained in the cycloheximide experiments. Conditioned medium collected early after injury (0–3.5 h) suppressed the proliferative response in other ganglia cultured for 27 h. This suggests that inhibitory molecules are released early during the injury process or that this signal is present during the uninjured state and its effects last for 3.5 h after explant culture. The finding that cycloheximide exposure to ganglia 0–3.5 h after injury blocks the inhibitory effect on glial proliferation further suggests that the inhibitory proteins in our cultures are both made and released during the first 3.5 h after injury. Ganglia cultured in conditioned medium collected from 0 to 27 h after injury enhanced the proliferation seen in other 27-h ganglia cultures. Our cycloheximide experiments demonstrated that blocking protein synthesis between 3.5 and 7 h after injury abolishes glial proliferation, and we conclude that this is the earliest time the mitogenic signal is produced.

It is unclear whether the production and the release of the mitogenic signal is continuous or transient. Scintillation counts of ganglia cultured for 48 h showed that proliferation continued to increase to approximately eight times that of 27-h cultures. Double labeling experiments using [^{3}H]thymidine and BrdU have revealed that by 48 h, one can find three different patterns of glial cell staining. There are two populations of satellite cells that incorporated the early proliferation marker and a large population that incorporated the late proliferation marker. It is important to note that proliferating satellite cells always outnumber proliferating Schwann cells, especially during the late portion of the 48-h culture period. Several interpretations of the large, late proliferating, satellite cell population are possible. First, satellite cells that incorporate the later proliferation marker could be activated by a higher concentration of the same glial mitogen that activates the earlier proliferating cells. Second, it is possible that the cells that produce the initial mitogen could produce another separate mitogen that activates satellite glial cells to proliferate. Third, the late proliferating satellite cells may have been signalled to proliferate early, but they may require a longer time to enter S phase. We do not favor the first of these explanations because experiments designed to collect a higher concentration of this putative glial mitogen failed to demonstrate any additional mitogenic effects. Conditioned medium collected between 0 and 48 h after injury demonstrate no additional facilitative effect on glial proliferation compared with 0–27-h unconditioned medium, suggesting that this late proliferating population is not dependent upon an increasing concentration of the same mitogen. The fact that no differences in proliferation were revealed by culturing with conditioned media collected from the incubation of one versus four ganglia also speaks against the late proliferating population being dependent upon an increasing concentration of the same mitogen. However, it remains possible that we did not reach a concentration of mitogen that was high enough to induce early proliferation by the population of the cells that incorporate [^{3}H]thymidine late in the 48-h culture period. Despite the lack of an additional effect on glial proliferation using the 48-h conditioned medium, the second interpretation (a separate, late mitogen) remains viable. A second diffusible mitogen could be released but may be degraded by 48 h in culture and therefore its effects on proliferation over an additional 0–27-h culture period would not be detected. Alternatively, the putative second mitogen may be membrane bound so that its effects would not be observed in conditioned medium.

By 48 h, the percentage of Schwann cells that are proliferating has also increased compared with earlier periods after injury. These proliferating Schwann cells, however, are concentrated proximal to the injury sites at the edges of cut stumps of the axonal tracks. Their activation may be the result of a different type of signal. This is consistent with previous reports of proliferating Schwann cells at sites of injury (Logan et al., 1953; Salzer and Bunge, 1980). The possible stimuli for Schwann cell proliferation are numerous. Axolemma- and myelin-enriched fractions can both be mitogenic for Schwann cells (Ratner et al., 1984, 1985; Salzer et al., 1980; Sobue and Pleasure, 1985; Yoshino et al., 1984; Dent et al., 1991, 1992). Furthermore, macrophages facilitate injury-induced Schwann cell proliferation (Baichwal et al., 1988; Yoshino et al., 1987). Although macrophages may be sufficient stimuli, it is unlikely that they are necessary for Schwann cell proliferation as few macrophages are detected in our trigeminal explant cultures.[1] Recent studies have identified the glial growth factor (GGF)/Neuregulin gene whose products have been shown to be potent mitogens for Schwann cells (Marchionni et al., 1993; Goodearl et al., 1993). Proteins from multiple transcripts of this gene have been shown to activate a 185-kD protein on Schwann cells throught to be the p185^{erbB2} receptor tyrosine kinase. In situ hybridization studies in embryonic mouse have revealed expression of GGF mRNA localized only to neurons with prominant expression in sensory ganglia, particularly the trigeminal ganglion (Marchionni et al., 1993; Orr et al., 1993). One possibility is that the release of GGF from injured trigeminal neurons also may serve as the mitogen inducing satellite cell proliferation.

The ability of exogenous growth factors to decrease the glial proliferative response in vitro in the present study may be a function of the neurotrophic growth factors which rescue injured neurons. The biochemical changes associated with axotomy are hypothesized to reflect the interruption in the retrograde transport of neuronal growth factors from targets (Rich et al., 1989). In vivo, it has been suggested that reinnervation returns the supply of NGF back to the previously injured neurons. NGF binds to *trk* A receptors to mediate its neurotrophic effects (Kaplan et al., 1991). In situ hybridization studies suggest that adult trigeminal sensory neurons appear capable of responding to multiple neurotrophins as evidenced by the heterogenous distribution of mRNAs coding for *trk* A, the NGF receptor, *trk* B, the BDNF and NT-4/5 receptor and *trk* C, the receptor for NT-3 (V. M. K. Verge, personal communication). Given that trigeminal sensory neurons express neurotrophin receptors, it is conceivable that replacing trophic factors (lost as a result of axotomy) would reduce the neuronal injury responses. A decrease in glial proliferation was seen in ganglia cultured in 50 ng/ml of either NGF or BDNF. The specificity of the action of NGF is presumably localized to neurons, given that only a sub-population of neurons, and not satellite cells, in the trigeminal ganglion express the *trk* A receptor (Verge et

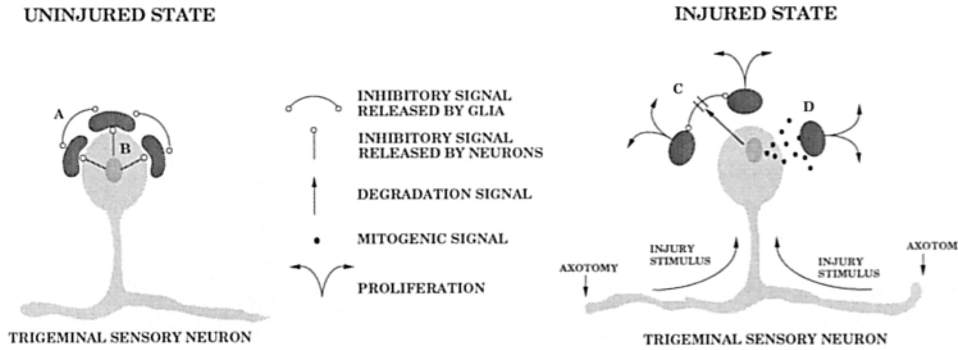

Figure 7. Models for injury-induced glial proliferation. In the uninjured state, there are glial inhibitory proteins produced by satellite cells (*A*) or by neurons (*B*) preventing satellite cells from proliferating. When neurons are axotomized, they produce a mitogenic protein that either indirectly (via an enzyme [*C*] that breaks down the glial inhibitory protein) or directly (via a mitogenic protein [*D*] which overcomes any inhibitory influence) causes satellite cells to proliferate.

al., 1992). In addition to to neuronal expression, hybridization signal for *trk* B mRNA could be detected over the satellite cells surrounding the trigeminal neurons (V. M. K. Verge, personal communication). However, it is not known at this time whether the trigeminal staining of *trk* B represents the $gp145^{trkB}$ (full length receptor) or the $gp95^{trkB}$ (cytoplasmically truncated receptors lacking signal transduction capability) (Klien et al., 1990). Neurotrophic receptors lacking cytoplasmic domains may pool trophic factors at certain sites, and may serve as transporters of these molecules to neurons (Chao, 1992). Until the form of the *trk* B receptor on satellite cells can be determined unequivocally, the decrease in satellite cell proliferation by BDNF should be treated cautiously. NGF and BDNF do cross react with both *trk* A and *trk* B receptors (Rodriguez-Tébar et al., 1990), however, this cross-reactivity is minimal at the concentration of growth factor we have used. It is possible that the lack of complete inhibition of the glial proliferative response after NGF or BDNF treatment may be due to the differential expression of trophic factor receptors in the trigeminal ganglion. The withdrawal of NGF from sympathetic and sensory ganglia results in atrophy and eventually cell death (Rich et al., 1984; Yip et al., 1984). It has been hypothesized that the lack of NGF turns on a cell death program which actively kills the cell. Neuronal survival can be prolonged if a protein synthesis inhibitor is added in vitro (Martin et al., 1988; Scott and Davies, 1990) and in vivo (Oppenheim et al., 1990), and thus NGF appears to be repressing the expression of death proteins. Although we have no evidence to suggest that explanted trigeminal sensory neurons are dying by 27 h, it remains possible that satellite cells are reacting to dying neurons and not to injury since the cell death process may be accelerated under in vitro conditions. The reduction of satellite cell proliferation with the additon of cycloheximide or neurotrophic factors seem to be consistent with this view. It is clear that trigeminal sensory neurons have been severely injured by explantation, but whether these neurons are actually dying remains to be determined.

The function of satellite cell proliferation resulting from injury is unclear. However, satellite cells have been shown to maintain neuronal metabolic requirements. During development, the presence of satellite cells greatly enhances the survival of ganglionic neurons in culture (Shimizu, 1968; Varon and Raiborn, 1972), and enhances axonal growth and maturation in embryonic cultures (Varon and Raiborn, 1972). Thus, satellite cells are capable of providing trophic support for neurons during development. Injury-induced satellite cell proliferation may help to serve a similar function. When neurons are injured, the metabolic requirements of these cells increase (Kreutzberg and Emmert, 1980; Smith et al., 1984). Satellite cells may proliferate to compensate for this increased requirement.

The possible interactions between neurons and glia during both the uninjured and injured states are schematized (Fig. 7). In the uninjured state, there is very little satellite glial proliferation. Injections of [^{3}H]thymidine in vivo reveal autoradiographic labeling of only a very small number of satellite cells.[1] These satellite cells may not normally proliferate because they are under inhibitory control. It is not clear whether the putative inhibitory signal is mediated by way of glial–glial or neuronal–glial interactions. The inhibitory signal that we have shown to exist could also arise from neurons (Fig. 7 *B*). In the enteric nervous system, neuronal–glial signals that inhibit glial proliferation seem to be dependent on contact-mediated mechanisms (Eccleston et al., 1989). However, the inhibitory signal that we have described is diffusible. Alternatively, the inhibitory signal may be glial–glial in nature. It is thought that the negative autocrine control of quiescent Schwann cells is due to enzymatic cleavage of fibronectin by Schwann cells via the activation of the plasminogen activator system (Muir and Manthorpe, 1992; Muir et al., 1990). It has been speculated further that a neuronal enzyme inhibitor or modulator of negative autocrine signals between Schwann cells may be responsible for the induction of Schwann cell proliferation during development (Muir and Manthorpe, 1992). We suggest that satellite glial cells are under negative control in the uninjured state, and that the induction of satellite cell proliferation during injury is caused by a neuronal protein that interferes either directly or indirectly with this inhibitory control. The inhibitory signal that is collected in the medium between 0 and 3.5 h after injury in explant culture could represent an inhibitory glial signal for proliferation passing between glial cells (Fig. 7 *A*) that is also constitutively present in the uninjured state.

In the injured state, neurons produce and release a mitogenic signal for satellite glial cells after their axons have been cut. There is no evidence to differentiate the possibility that

the mitogenic and inhibitory signals interact directly (Fig. 7 *C*) or independently of each other (Fig. 7 *D*). In Fig. 7 *C*, the mitogenic signal could be an enzyme which breaks down an inhibitory signal for glial proliferation released by glial cells. The breakdown of this inhibitory signal would result in mitogenesis of glial cells. In Fig. 7 *D* the mitogenic signal acts directly on glial cells to overcome the negative effects of the inhibitory signal, or induces glial cells to stop the production of this inhibitory protein. In both of these models, glial proliferation could be stopped with the termination of the production of the neuronal signal.

The evidence from this study shows that injured neurons participate in the induction of satellite cell proliferation in the trigeminal ganglion. Diffusible glial mitogenic and inhibitory signals are produced and released which dynamically control glial proliferation. The production of the mitogenic factor appears to be regulated by target derived trophic agents since the addition of trophic factors in vitro reduces this glial proliferative response. Hopefully the isolation and identification of both mitogenic and inhibitory proteins will facilitate the understanding of the signaling mechanisms involved between neurons and glia during injury.

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