

# TGF $\beta$ Trophic Factors Differentially Modulate Motor Axon Outgrowth and Protection from Excitotoxicity

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**Transforming growth factor (TGF)  $\beta$ -like trophic factors have been shown to be protective in acute neuronal injury paradigms. In the current study, we analyzed and compared members of this growing family, including glial cell line-derived neurotrophic factor (GDNF), neurturin, nodal, persephin, and TGF $\beta$ 1, for protection against chronic glutamate toxicity. In parallel, we developed a organotypic spinal cord culture system to study the ability of these factors to promote motor axon outgrowth across white matter. Using these systems, we were able to differentiate the neuroprotective effect of the TGF $\beta$ -like factors from their motor axon outgrowth-promoting activity. GDNF, neurturin, persephin, nodal, and TGF $\beta$ 1 all protected against excitotoxic motor neuron degeneration. Low amounts of GDNF (1 ng/ml) and high concentrations of neurturin induced vigorous motor axon outgrowth. In contrast, nodal, persephin, and TGF $\beta$ 1 did not induce motor axon outgrowth. Both GDNF and neurturin bind to Ret receptor complexes and were capable of activating the MAP kinase pathway. A specific inhibitor of MAP kinase, PD98059, inhibited the motor axon outgrowth-promoting activity of the GDNF but not the neuroprotective activity. Similarly, the specific PI3K inhibitors, LY294002 and wortmannin, were able to inhibit the promotion of motor axon outgrowth by GDNF, but did not affect neuroprotective activity. Our results suggest that the neurite outgrowth-promoting effect of GDNF is mediated through the PI3K and MAP kinase pathways. The neuroprotective effect of GDNF appears to be through a separate pathway.** © 2000 Academic Press

**Key Words:** amyotrophic lateral sclerosis; TGF $\beta$ ; GDNF; persephin; neurturin; nodal; axon outgrowth; motor neuron; MAP kinase; PD98059; PI3K; LY294002; wortmannin.

## INTRODUCTION

Neurotrophic factors play an important role in survival, development, and maintenance of the nervous system. Some factors can protect neurons against acute

and chronic insults and some are able to promote neurite outgrowth. Understanding the cellular mechanisms responsible for these effects may be important in developing treatment for many neurological diseases.

Multiple transforming growth factor  $\beta$  (TGF $\beta$ )-like neurotrophic factors have been identified recently, including glial cell line-derived neurotrophic factor (GDNF), neurturin, nodal, and persephin (20–22, 49). GDNF exhibits trophic effects on central dopaminergic neurons, spinal cord motor neurons, forebrain cholinergic neurons, cerebellar Purkinje cells, and dorsal root ganglia (5, 13, 17, 23, 42, 50) and protects them from a variety of insults (1–3, 18, 27). GDNF also promotes fiber outgrowth of fetal ventral mesencephalic grafts in animal models of Parkinson's disease (40).

Neurturin is a neurotrophic factor that shares 42% homology with GDNF (20). Like GDNF, neurturin supports superior cervical ganglia, nodose neurons, and dorsal root ganglia. Persephin was recently cloned based on homology with the GDNF and neurturin sequences (22). Persephin promotes survival of ventral midbrain dopaminergic neurons in culture and prevents their degeneration after 6-hydroxydopamine (OHDA) treatment *in vivo* (22). It supports motor neurons *in vitro* and after sciatic nerve axotomy (22). Finally, nodal, another newly described member (49), appears to be essential for the formation of asymmetric left–right body axis (9).

In amyotrophic lateral sclerosis (ALS), chronic excitotoxicity is thought to contribute to the motor neuron degeneration (37, 38). Chronic excitotoxicity due to the loss of glutamate transport can be modeled *in vitro* by using organotypic spinal cord cultures and has been shown to produce slow degeneration of motor neurons (33). This system has been used as a preclinical screen for potential neuroprotective agents; for example, riluzole, insulin growth factor (IGF-1), and gabapentin were protective, whereas ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) failed to protect against chronic motor neuron degeneration (10, 35).

In this study, we modified our organotypic spinal cord culture model to investigate the ability of different trophic factors to induce motor axon outgrowth across the surrounding white matter. We studied the new members of the growing family of TGF $\beta$ -like trophic factors, GDNF, nodal, neurturin, and persephin, for their ability to protect motor neurons against chronic glutamate toxicity and to promote motor axon outgrowth. All these factors were protective of motor neurons against chronic glutamate toxicity, but only GDNF and neurturin were able to induce motor axon outgrowth across the surrounding white matter. This effect on motor axon outgrowth may be mediated through the MAP kinase and PI3K pathway.

## METHODS

### *Materials*

Rodent neurturin and persephin were prepared as previously described (12). GDNF was supplied by Amgen (Thousand Oak, CA). Human neurturin and persephin were provided by Genentech (San Francisco, CA). All peptide factors were dissolved in phosphate-buffered saline. Each week, fresh media were prepared with newly thawed factors. In all cases, the vehicle never exceeded 1% (v/v) of the final culture medium. (D,L)-Threohydroxyaspartate (THA), LY294002, and wortmannin were obtained from Sigma Chemical Company (St. Louis, MO). All other reagents were culture grade.

### *Organotypic Spinal Cord Cultures*

Organotypic spinal cord cultures were prepared from lumbar spinal cords of 8-day-old rat pups, as described previously (33). Lumbar spinal cords were collected under sterile conditions and sectioned transversely into 350- $\mu$ m slices with a McIlwain tissue chopper. Slices were cultured on Millicell CM semipermeable culture inserts at a density of five slices/well in an incubator at 37°C (5% CO<sub>2</sub>, 95% humidity). Under these conditions, >95% of cultures retained cellular organization, and a stable population of motor neurons survived for more than 3 months. Culture media (50% minimal essential medium and Hepes (25 mM), 25% heat-inactivated horse serum, and 25% Hanks' balanced salt solution (Gibco) supplemented with D-glucose (25.6 mg/ml) and glutamine (2 mM), at a final pH of 7.2) were changed twice weekly. Drugs and factors were added when media were changed. No drugs were added for the first 7 days after culture preparation. In most cases, trophic factors were added either alone or in combination with THA (100 $\mu$ M) for 4 weeks.

### *Identification of Motor Neurons*

Motor neurons were identified immunochemically by two independent markers: SMI-32 (an anti-nonphosphorylated neurofilament antibody) and in some cases

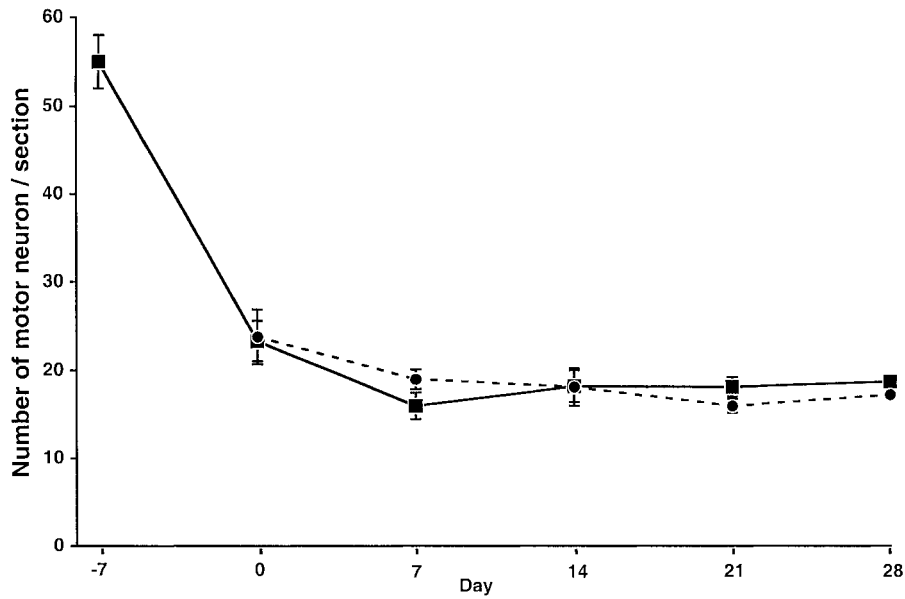
the motor neuron-specific marker islet-1. These markers provided more reliable indices of motor neurons than the previously used biochemical assays for choline acetyltransferase (33). Lower motor neurons were visualized in cultures with monoclonal antibodies, SMI-32 (Sternberger Monoclonals Incorporated, Baltimore, MD) and in some cases verified with anti-Islet-1 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA). Cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min and then permeabilized with cold methanol or 0.1% Triton X-100 for SMI-32 and islet-1 staining, respectively. Cultures were incubated with monoclonal SMI-32 antibodies (1:8000) or monoclonal islet-1 antibodies (1:100) overnight at 4°C. After incubation with biotinylated horse anti-mouse antibodies and ABC reagents (Vector Labs), the standard diaminobenzidine (DAB) reaction was used for color development. SMI-32 specifically stains non-phosphorylated neurofilaments, which are abundant in motor neuron cell bodies and can be readily used to identify motor neurons by morphological criteria. Motor neurons were quantified using the following three criteria: immunostaining of SMI-neurons with SMI-32, size >25  $\mu$ m, and localization to the ventral gray region of spinal cord slices. The motor neuron count assessed by SMI-32 was verified by islet-1 staining in some cases. Counts of SMI-32-stained large ventral horn neurons agreed closely with motor neuron counts obtained from counts of serially sectioned slices and from retrograde labeling of DilC18 (Molecular Probes, Eugene, OR) (33).

### *Motor Axon Outgrowth*

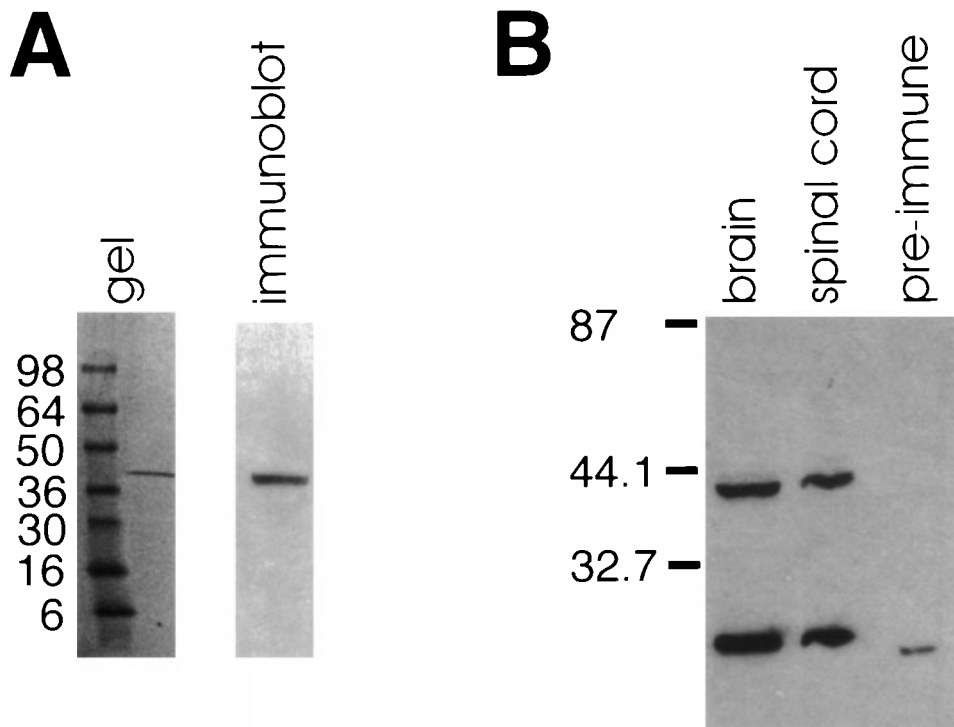
To assess motor axon outgrowth, we modified the organotypic spinal cord cultures described above by coating the culture inserts with collagen (5 $\mu$ g/cm<sup>2</sup>) and treated with selective trophic factors. Neurite outgrowth was seen and quantified by immunostaining for neurofilaments by using SMI-32 antibody. Neurite growth was quantified by counting the number of fibers exiting the spinal cord slices after 2 weeks in culture. In some experiments, a specific MAP kinase kinase (MEK) inhibitor, PD98059, and PI3K inhibitors, LY294002 and wortmannin, were added at various concentrations.

### *Cloning and Expression of Nodal*

To study the effect of nodal, we first cloned and expressed murine nodal. Nodal was cloned by PCR by using an N-terminal primer, CCCGTCGACATGAGT-GCCCACAGCCTCCGC, and a C-terminal primer, CCCCTCGACTCAGAGGCACCCACACTCCTC, which were designed to include *SalI* and *XhoI* sequence sites for cloning. After the sequence of the cloned product was confirmed, it was ligated into pFastBac HTc. Positive colonies from transformed BRL SE cells were identified by restriction digestion, followed by genera-



**FIG. 1.** Motor neurons identified by SMI-32 were stable for more than 4 weeks when cultured with either standard media (solid squares) or media containing GDNF (10 ng/ml, solid circles).



**FIG. 2.** Cloning and detection of nodal protein. (A) Mouse nodal was cloned and expressed in a baculovirus expression system. The growth and purification of nodal in sf9 resulted in a single band on Coomassie blue-stained polyacrylamide gel. The expressed protein was purified by a nickel column and was more than 95% pure (gel) and represented nodal protein by immunoblot. (B) Nodal protein was detected in brain and spinal cord immunoblots by using affinity-purified, oligopeptide antibodies to nodal. The antibodies recognized the predicted 38-kDa protein. The lower MW band may be fragments of nodal, as it was not seen with peptide blocking controls (not shown).

tion of recombinant bacmid from transposed DH10Bac cells. The nodal containing recombinant bacmid was transfected by lipofection into sf9 cells. The titer of the nodal lipofection supernatant fraction was expanded by infection of a T-25 flask of sf9 cells. A 3-liter volume was subsequently prepared and after 72 h, cells were centrifuged and lysed. The nodal protein was purified over a nickel-NTA resin column at 4°C.

For detection of nodal protein, an oligopeptide from the presumed mature region of nodal (K<sup>324</sup> to L<sup>339</sup>) was used to generate rabbit polyclonal antibodies. The HPLC-purified peptide was then coupled to thyroglobulin by using glutaraldehyde. Antisera were generated against the protein-conjugated synthetic peptides in New Zealand White rabbits (Covance, Denver, PA) as previously described (36). The crude antisera were affinity purified on a column prepared by coupling bovine serum albumin-conjugated nodal peptide to Affi-Gel 15 (Bio-Rad, Rockville Center, NY). Sera con-

taining nodal-immunoreactive antibody were confirmed on immunoblots containing sf9 cell lysates, rat brain, and spinal cord.

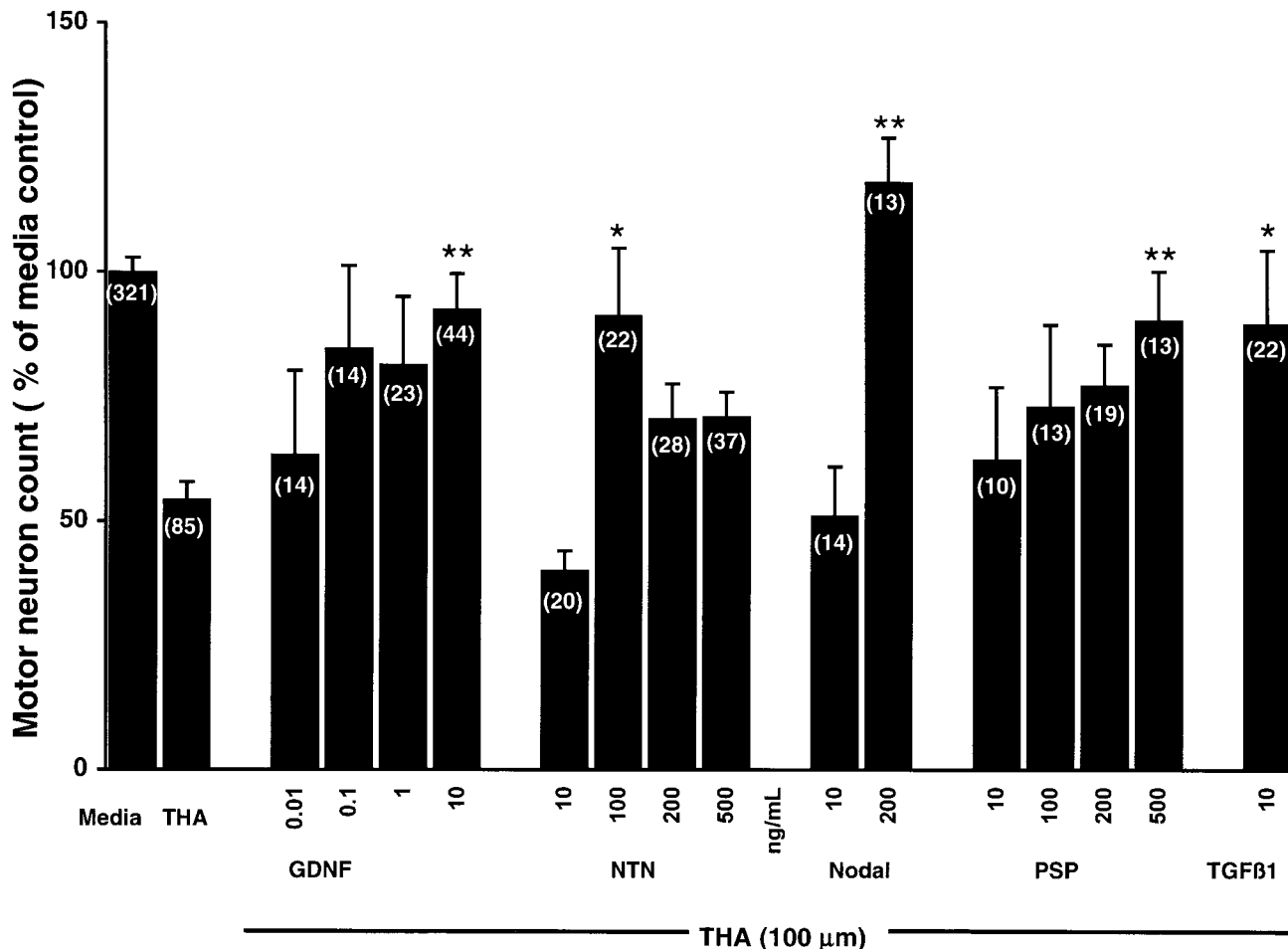
### Statistical Analysis

All experiments were replicated two to three times and yielded reproducible results. Results are expressed as means  $\pm$  SEM. Intergroup differences were determined by ANOVA with Tukey's post hoc comparison to compare between all groups. (Statview, SAS Institute, Cary, NC). Differences were considered significant at  $P < 0.05$ .

## RESULTS

### Identification of Motor Neurons in Organotypic Spinal Cord Cultures

Motor neurons in organotypic cultures remained viable for more than 3 months. In this culture system,



**FIG. 3.** TGF $\beta$ -like trophic factors can protect against THA-induced chronic glutamate toxicity. After 4 weeks of culture, the number of motor neurons in THA-treated cultures dropped by approximately 50% compared to control. GDNF at concentrations at 10 ng/ml protected against THA toxicity. Neurturin (NTN) was protective at 200 ng/ml, while nodal and persephin (PSP) required higher doses to achieve neuroprotection. TGF $\beta$ 1 was protective at 10 ng/ml. Statistical significance: \*\* $P < 0.01$  and \* $P < 0.05$ , when compared to THA-treated cultures. Numbers of spinal cord sections in the experiment are shown in parentheses.

motor neurons can easily be identified both by their morphology (size  $>25\mu\text{m}$ , large axon) and by their localization to the ventral horn in the spinal cord with SMI-32 immunostaining. When studied over 4 weeks, cultures were shown to maintain a stable population of approximately 18–20 motor neurons (Fig. 1). This is comparable to the counts of individual motor neurons obtained previously by either choline acetyl transferase immunocytochemistry or counts of large ventral horn neurons seen in serial semithin plastic sections of the cultures (33).

#### Cloning and Expression of Nodal

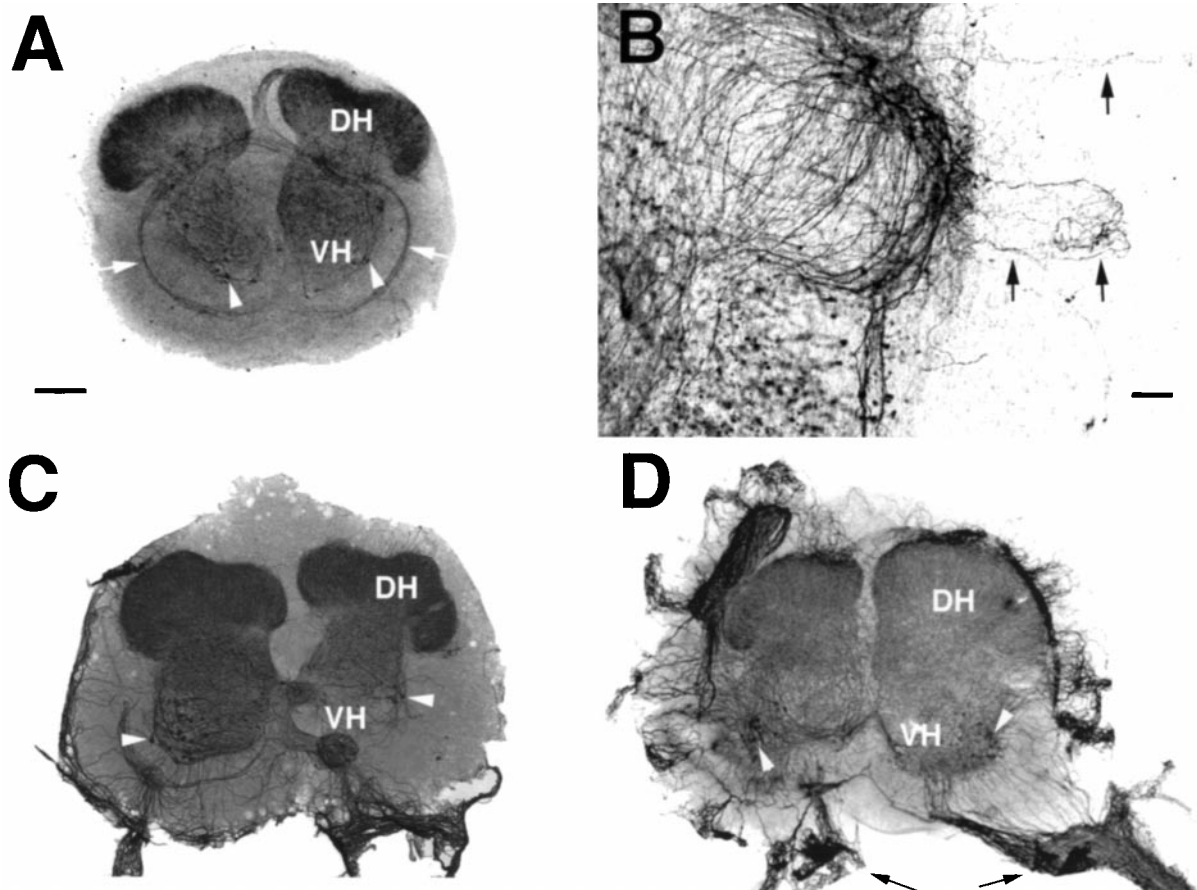
With use of the BRL bac-to-bac baculovirus system, nodal was produced in sf9 and resulted in a single band on polyacrylamide gel after purification (Fig. 2A), which when immunoblotted was reactive with anti-nodal antibody. The anti-nodal antibody did not recognize other TGF $\beta$ -like trophic factors including GDNF, NTN, and persephin (results not shown). This resulted in the

purification of approximately 1–2 mg of  $>95\%$  pure nodal protein from each 3-liter sf9 harvest.

On the basis of nested RT-PCR studies (first PCR primers: nodal F1, CTTCAACCTGATGGCTGG; nodal R1, GGAGTTCATCAGCATTGTG; second PCR primers: nodal F2, GCTCCTGGATCATC; nodal R2: GCAAGCCAA-TTCCAGCAC), nodal was detected in brain and spinal cord (not shown). With the affinity-purified oligopeptide antibody to nodal, immunoblots of rat whole brain or spinal cord homogenates ( $20\mu\text{g}/\text{lane}$ ) revealed a 38-kDa band, the predicted size for nodal (Fig. 2B). A second lower molecular band was also observed, which may be comprise of proteolytic fragments of nodal, as it was not seen with peptide blocking controls (not shown).

#### Chronic Glutamate Toxicity Can Be Blocked by TGF $\beta$ Superfamily of Trophic Factors

None of five trophic factors, GDNF, neurturin, persephin, nodal, and TGF $\beta$ 1, appeared to have any



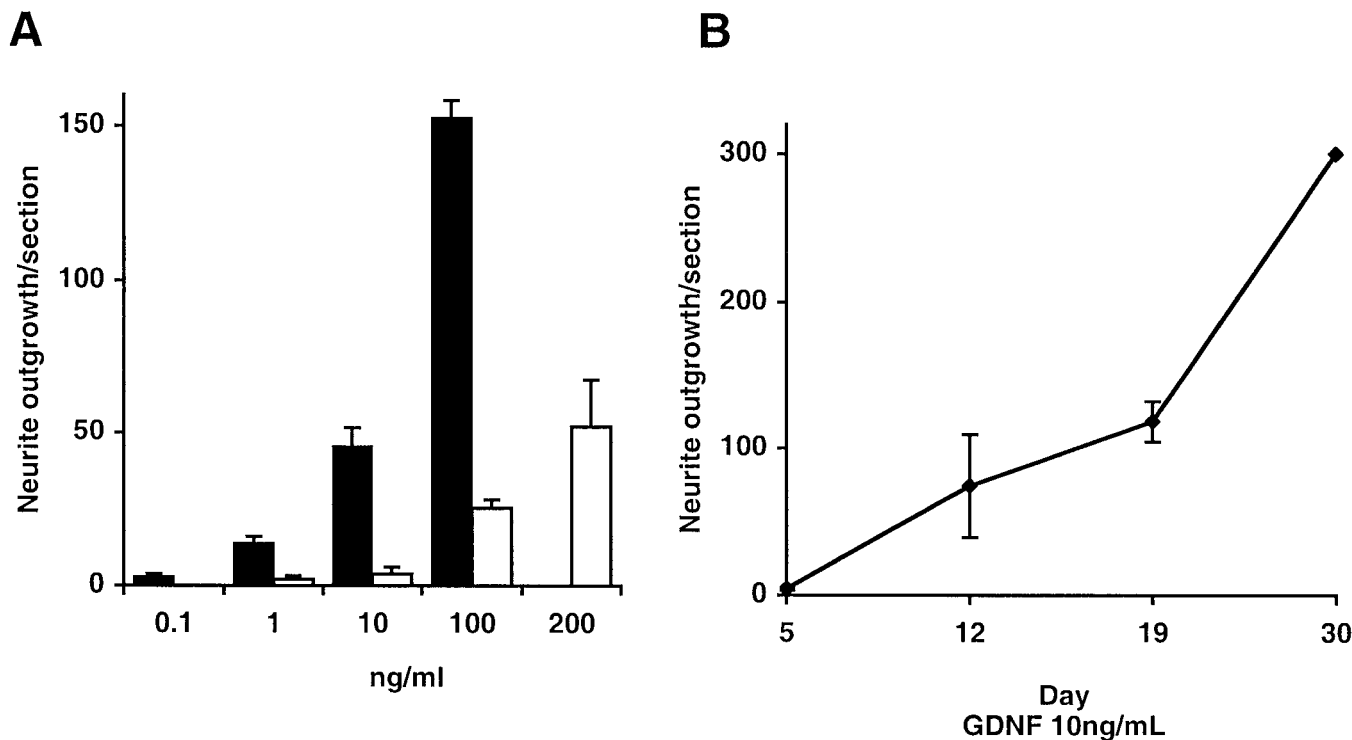
**FIG. 4.** Effect of GDNF (10 ng/ml) on organotypic spinal cords grown on collagen-coated culture inserts. (A) Spinal cord after 4 weeks of culture with medium alone. Motor axons remained confined to the gray matter or at the margin between the gray and white matter (white arrows). (B) With addition of GDNF (10 ng/ml), neurites (black arrows) stained by SMI-32 can be seen exiting the spinal cord culture as early as 5 days. (C) At 19 days of culture, abundant numbers of large caliber neurites, mostly derived from the ventral motor neuron pool, can be seen exiting the ventral roots. Some fibers wrapped around the spinal cord and grew toward the dorsal horn. At higher power, only a few fibers appear to be derived from dorsal horn. (D) Similar results are shown at 30 days of culture. White arrowheads, motor neurons; VH, ventral horn; DH, dorsal horn. Bars,  $250\mu\text{m}$  in A, C, and D;  $100\mu\text{m}$  in B.

intrinsic toxicity when added to spinal cord cultures for up to 4 weeks (data not shown). All five factors significantly protected motor neurons against toxicity resulting from blockade of the glutamate transporter with THA (Fig. 3). GDNF and TGF $\beta$ 1 appeared to be the most potent trophic factor against THA toxicity, with neuroprotective activity at concentrations as low as 10 ng/ml. Neurturin was protective at 100 ng/ml. However, at higher doses, the protective effect became insignificant. Persephin prevented excitotoxic motor neuron degeneration in a dose response manner starting at 500 ng/ml. Rat and human neurturin and persephin were equally neuroprotective. Nodal was also significantly neuroprotective at a relatively high dose of 200 ng/ml. As a comparison, several non-TGF $\beta$  neurotrophic factors were not found to be protective including BDNF (1–100 ng/ml), NT4–5 (1–50 ng/ml), CNTF (1–100 ng/ml), leukemia inhibitory factor (LIF, 10–100 ng/ml), and cardiotropin-1 (1–100 ng/ml) (results not shown).

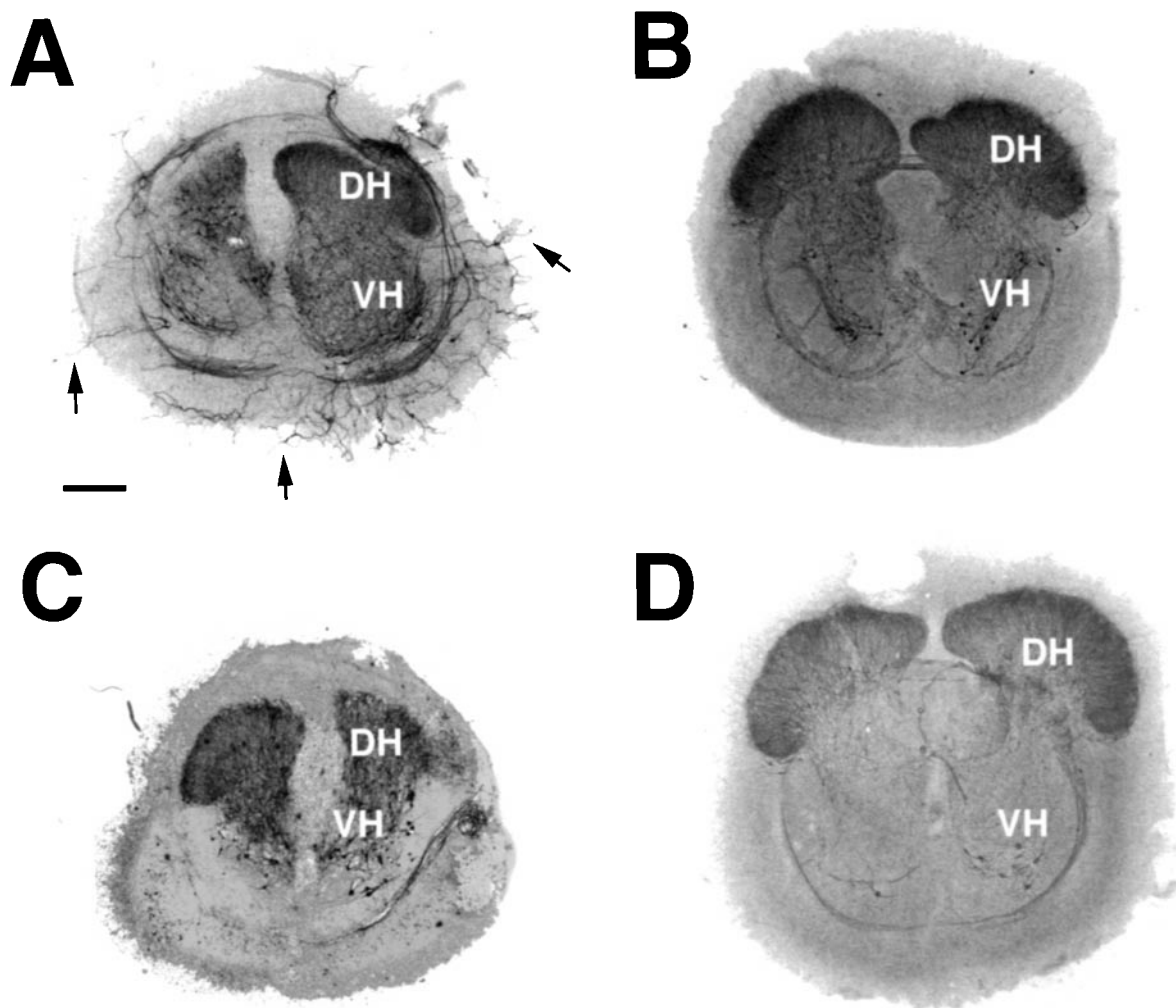
#### Only GDNF and Neurturin Are Able to Induce Motor Axon Outgrowth

Under standard culture conditions, motor axons remained confined to the gray matter or at the margin between the gray and white matter that comprised the original spinal cord *in vivo* (Fig. 4A, white arrows).

However, when culture inserts were first coated with collagen (5 $\mu$ g/cm<sup>2</sup>) or laminin (20 $\mu$ g/ml) and spinal cord cultures were then treated with GDNF, vigorous outgrowth of motor axons was observed. The motor axons were able to “penetrate” the white matter and even exit the spinal cord slice to re-form ventral root-like structures. Without collagen coating, the outgrowth continued to exit the spinal cord, but tended to grow by wrapping around the edge of the spinal cord without extending on to the insert. This growth occurred as early as 5 days after treatment with GDNF (Fig. 4B) and continued for the entire 4-week culture period (Fig. 5B). By tracing back to their neuronal cell bodies, more than 90% of all neurites were derived from the large ventral horn motor neurons (Figs. 4C and 4D). Without a clear target, these neurites tended to branch and to grow into a matted field of neurites. The response could be seen with doses of GDNF as low as 1 ng/ml. Increased doses of GDNF produced a greater outgrowth of axons (Fig. 5A, black bars). Neurturin was also able to promote motor axon outgrowth (Fig. 6A). This effect required higher doses, however, with significant effects starting at 100 ng/ml (Fig. 5A, open bars). Neither persephin, nodal, nor TGF $\beta$ 1 up to 500 ng/ml had any significant effect on neurite outgrowth (Figs. 6B–6D). Similarly, other non-TGF $\beta$ -like factors failed to pro-



**FIG. 5.** (A) Effect of GDNF (black bar) and neurturin (open bar) on neurite outgrowth at 2 weeks of culture. GDNF induced neurite outgrowth at doses as low as 1 ng/ml. In comparison, a higher concentration of neurturin (100 ng/ml) was needed to induce neurite outgrowth. (B) GDNF neurite outgrowth continued and increased over time.



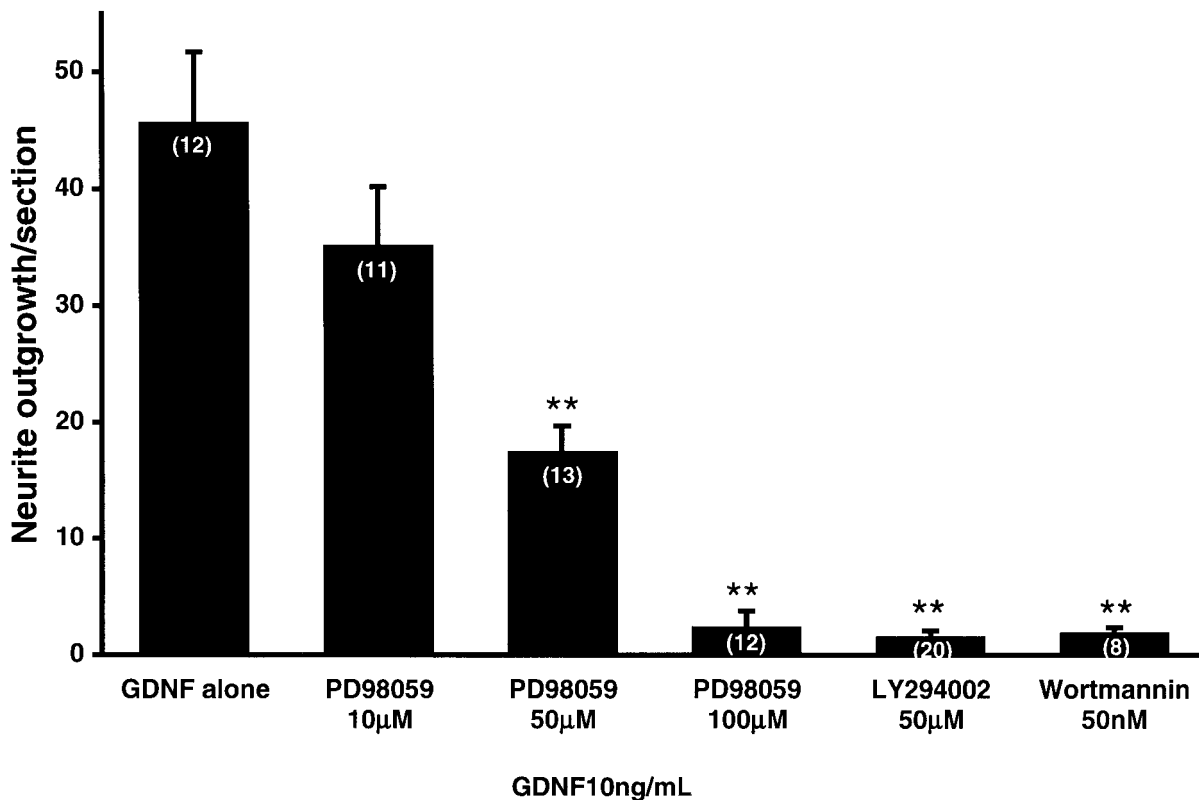
**FIG. 6.** Effect of other TGF $\beta$ -like trophic factors on neurite outgrowth. (A) Only neurturin at 100 ng/ml was able to induce neurite outgrowth. Other trophic factors nodal (B; 200 ng/ml), persephin (C; 500 ng/ml), and TGF $\beta$ 1 (D; 10 ng/ml) did not induce neurite outgrowth. Bar, 250 $\mu$ m.

note significant motor axon growth in this culture model, including IGF-1, BDNF, CNTF, or LIF at doses up to 100 ng/ml (results not shown).

*The PI3K and MAP Kinase Pathways Are Implicated in Induction of Motor Axon Outgrowth but Not for Neuroprotection by GDNF*

Both GDNF and neurturin were able to induce motor axon outgrowth and prevent excitotoxic motor neuron degeneration. Among the potential common pathways shared by these two factors is that both can bind to the Ret receptor complex and activate the PI3K and MAP kinase. To determine if the PI3K and MAP kinase pathways are central to the biological actions of GDNF both in promoting motor axon growth and in neuroprotection, PD98059, a specific MEK inhibitor, and LY294002 and wortmannin, specific PI3K inhibitors,

were used in both the excitotoxicity protection and the neurite outgrowth assays. PD98059 was able to block the promoting effect of GDNF on motor axon outgrowth at doses of 10 $\mu$ M and 50 $\mu$ M (Fig. 7). However, PD98059 did not prevent neuroprotection by GDNF in the model of chronic excitotoxicity (Fig. 8). In the chronic glutamate toxicity model, GDNF was able to protect motor neurons against THA toxicity (Fig. 9A) and induced some motor axon outgrowth (Fig. 9A, inset) in the presence of the lower concentration of PD98059 (10 $\mu$ M). When the higher concentration of PD98059 (50 $\mu$ M) was used, however, many motor neurons still survived THA toxicity, but only a few neurites grew out of the spinal cord (Fig. 9B). Similarly, the PI3K inhibitors, LY294002 and wortmannin, were able to inhibit the GDNF-induced neurite outgrowth (Fig. 7). Their effect appeared to be even more potent than the effect of PD98059. Like PD98059, the neuroprotective effect of



**FIG. 7.** Effect of MEK inhibitor PD98059 on neurite outgrowth and chronic glutamate toxicity. PD98059 was able to block GDNF (10 ng/ml)-induced neurite outgrowth in a dose-dependent fashion. At 50 $\mu$ M, neurite outgrowth was inhibited to approximately 40% of the GDNF-treated cultures. Similarly, PI3K inhibitors, LY294002 and wortmannin strongly inhibit neurite outgrowth. Statistical significance: \*\* $P < 0.01$  when compared to cultures without inhibitors. Numbers of spinal cord sections in the experiment are given in parentheses. Drugs and growth factors were changed twice weekly.

GDNF was not affected in the presence of these PI3K inhibitors (Fig. 8).

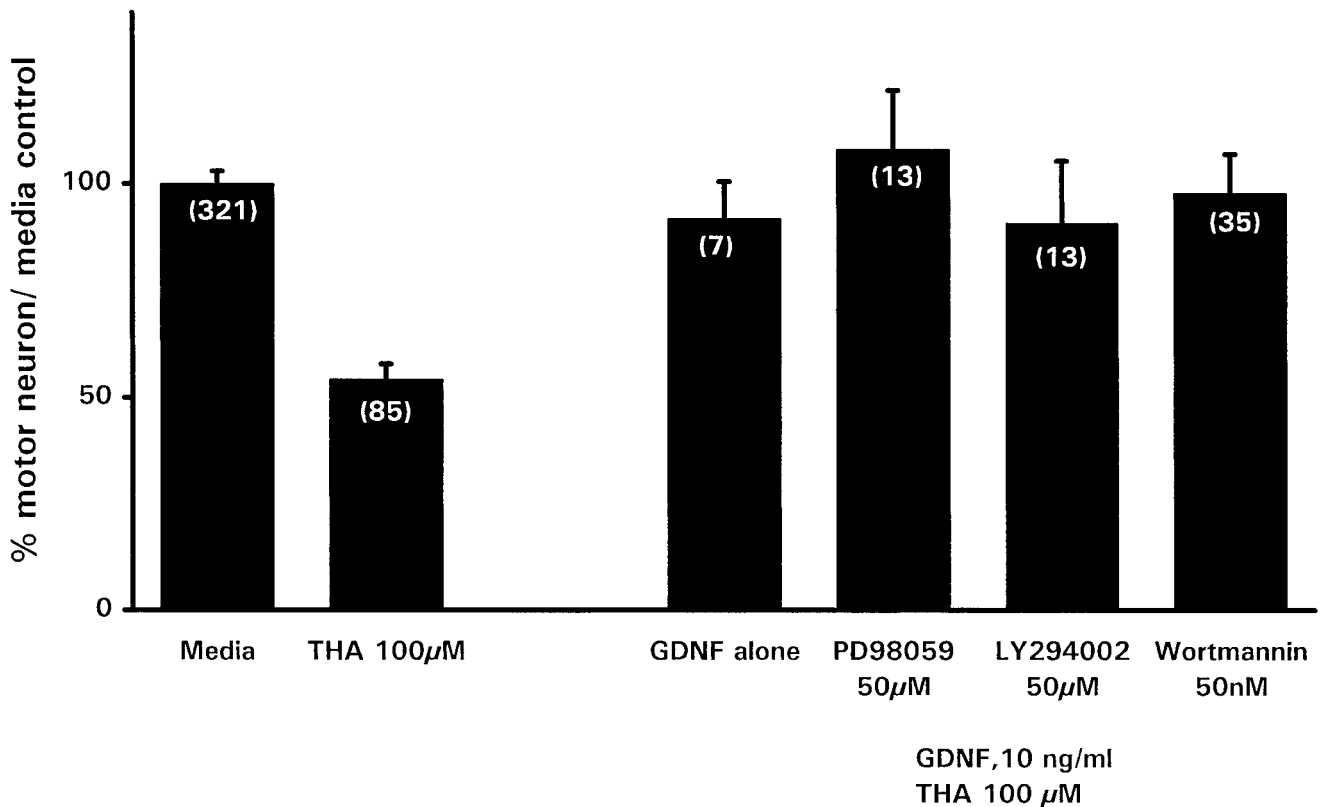
## DISCUSSION

In this study, we developed a novel model by modifying our excitotoxicity paradigm evaluate the potential of a trophic factor to promote motor axon outgrowth. Using these models, we were able to distinguish the neuroprotective property of TGF $\beta$ -like trophic factors from their effect on induction of motor axon outgrowths across the white matter. We found that TGF $\beta$ -like factors can protect motor neurons against chronic glutamate toxicity, although their efficacy varied dramatically. GDNF and TGF $\beta$ 1 appeared to be the most potent among the trophic factors studied, followed by neurturin, nodal, and then persephin. Only two of the trophic factors studied, GDNF and neurturin, were able to induce motor axon outgrowth across the white matter. Inhibitors of PI3K and MAP kinase pathways were able to block the neurite outgrowth, but not neuroprotection by GDNF, suggesting that the mechanisms for motor axon outgrowth across the white

matter may be distinct from the mechanism responsible for neuroprotection.

### *Outgrowth of Motor Axons*

Under normal culture conditions, the majority of neurites encircle the gray–white junction and do not cross the white matter, even when culture inserts were coated with collagen or laminin. Only GDNF and neurturin induced motor neurite outgrowth across the white matter in our system. GDNF is known to promote neurite outgrowth in dopaminergic neurons after 6-OHDA lesions (40) and in peripheral sympathetic neurons. GDNF produced robust, bundle-like, fasciculated outgrowth from chick sympathetic ganglion explants (45). Administration of GDNF enhanced sciatic nerve regeneration measured by the nerve pinch test (24). In contrast, Sagot *et al.* found that GDNF treatment on *pnn/pnn* mice significantly reduced the loss of facial motor neurons but found no effect on nerve degeneration (39). Studies of central nerve system regeneration have shown that many factors, such as myelin-associated glycoprotein (MAG), in the white matter hampers axonal outgrowth. One possible expla-



**FIG. 8.** The neuroprotective effect of GDNF against chronic glutamate toxicity was not inhibited by PD98059, LY294002, or wortmannin. Parentheses, number of spinal cord sections in the experiment. Drugs and growth factors were changed twice weekly.

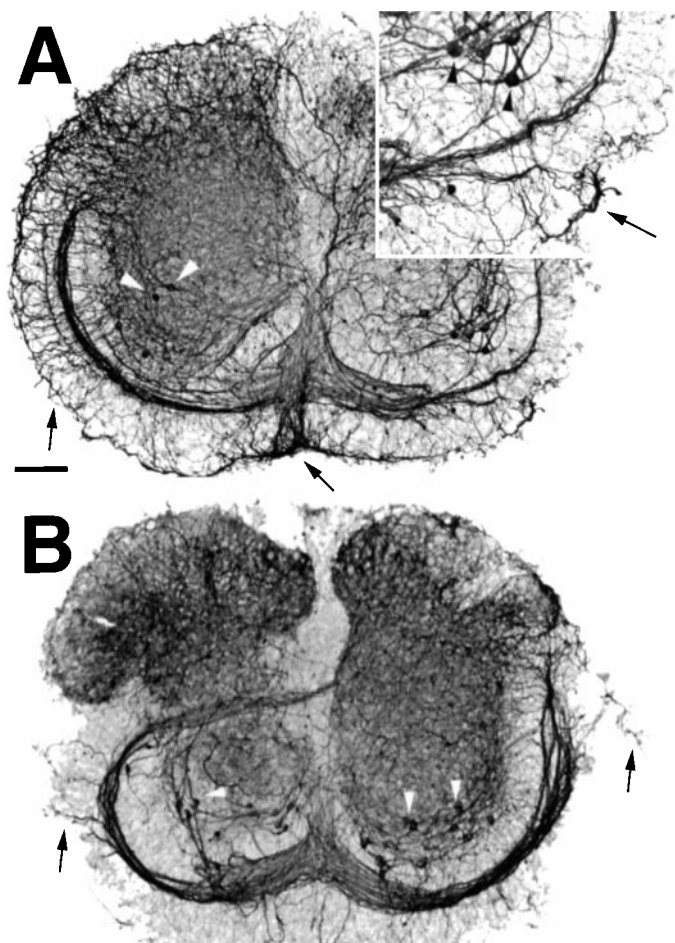
nation of our result may be that treatment with GDNF and neurturin allows axon to overcome the inhibitory effect of the white matter and grow across the white matter and exit the spinal cord. Our results are similar to that of Cai and co-workers in that priming cerebellar neurons with BDNF or GDNF, but not NGF, blocks inhibition of neurite outgrowth by MAG and myelin (6).

Little is known about the potential for neurturin to promote neurite outgrowth. Like GDNF, neurturin promotes survival of sensory neurons of the nodose and dorsal root ganglia and sympathetic neurons (20). What accounts for this shared property of only two of the TGF $\beta$  factors studied? Both GDNF and neurturin require the Ret receptor complex to transduce their physiological effects. The Ret receptor complex is a tyrosine kinase that can activate both MAP kinase and PI3K pathways (20, 26). Inhibitors such as LY294002 and wortmannin inhibited neurite outgrowth without interfering with neuronal protection afforded by GDNF. Stimulation by GDNF has been shown to result in ret tyrosine phosphorylation followed by Shc phosphorylation and Ras and ERK2 activation in SK-N-MC cells stably transfected with a full-length Ret construct (46). In PC12 cells, both MAP kinase and PI3K pathways play an important role in neurite outgrowth. MAP kinase activation has been shown to be important in

mediating the neurite outgrowth activity of NGF. Activation of MAP kinase is required for NGF to induce neurite outgrowth (15, 28). Inhibition of MAP kinase retards neurite outgrowth without affecting the ability of NGF to support PC12 cells (11, 28) and sympathetic neurons (47). PI3K inhibitors block neurite outgrowth (19). Our study shows that PI3K and MAP kinase pathways are also essential in the induction of neurite outgrowth by GDNF.

#### *Protection against Chronic Glutamate Toxicity*

Glutamate metabolism is thought to play an important role in the pathogenesis of ALS (29, 34, 38). It has been hypothesized that excess glutamate could contribute to chronic motor neuron degeneration. The organotypic spinal cord culture paradigm has been used to evaluate the role of multiple different insults on motor neurons in a system that effectively recapitulates the "normal" synaptic environment *in vitro*. Glutamate transport can be competitively inhibited by THA. By producing a persistent loss of glutamate transport with use of THA, mimicking the loss of transport present in ALS patients and transgenic mice (4, 31, 37), a slow excitotoxic degeneration of motor neurons occurs (33). Neurotoxicity in this model is likely to be secondary to



**FIG. 9.** Organotypic spinal cord cultures treated with THA (100 $\mu$ M), GDNF (10 ng/ml), and MEK inhibitor, PD98069. (A) In the presence of a low dose 10 $\mu$ M PD98059, GDNF protected motor neurons (white arrowheads; inset, black arrowheads) against chronic glutamate toxicity. Many neurites can be seen growing out of the spinal cord even though the inserts were not coated with collagen (inset, black arrows). (B) With a higher concentration of PD98059 (50 $\mu$ M), GDNF continued to protect motor neurons (white arrowheads) against chronic glutamate toxicity. Only a few neurites can be seen growing out of the spinal cord (black arrow). Bar, 250 $\mu$ M.

chronic elevations of extracellular glutamate resulting from inhibition of glutamate transport by THA, as well as the exchange of intracellular glutamate by carrier-exchange transport of THA. Accumulating data suggest that normal glutamate transport can modify normal synaptic currents. Chronic loss of glutamate transport, through antisense inhibition of transporter systems or gene knockout strategies, clearly demonstrates the essential role for transport in preventing chronic excitotoxic degeneration of neurons, including motor neurons (32). Our culture system provides a preclinical screening method for the increasing numbers of drugs postulated for clinical trials in motor neuron disease and a model to evaluate the mechanisms of chronic glutamate toxicity. In this model, riluzole, a drug that

increases survival of ALS patients and of transgenic mice with ALS SOD1 mutations, also protects motor neurons from dying. Using this paradigm, we have shown that the most potent neuroprotectants are agents that can inhibit glutamate release (riluzole, tetrodotoxin), glutamate synthesis (methionine sulfoximine, gabapentin), or block non-NMDA receptors (CNQX, BBQX, GYKI-52466) (35). It now appears that TGF $\beta$  factors, especially GDNF, are also potent neuroprotectants from chronic excitotoxicity.

The mechanism for GDNF neuroprotection from excitotoxicity is not clear, but it is shared by most of the TGF $\beta$  factors. Our data suggest that it may be mediated by a pathway independent of PI3K and MAP kinase activation, as inhibitors of these pathways have no effect on neuroprotection by GDNF. However, we cannot exclude the possibility that outgrowth of neurites is inhibited by these drugs first than neuroprotection. Several possible mechanisms could underlie GDNF neuroprotection: alterations of glutamate receptor subunits, induction of antioxidant enzymes to diminish oxidative stress secondary to excitotoxicity, or induction of glutamate transporters. Indeed, recent data suggest that certain neuronal factors can induce glutamate transporters (43). In addition, preliminary studies suggest that GDNF, neurturin, and persephin can induce synthesis of the glutamate transporter (16). Alternatively, TGF $\beta$ 1 has been shown to prevent neuronal Ca<sup>2+</sup> overloading of rat hippocampal neurons in response to NMDA or the Ca<sup>2+</sup> ionophore 4-Br-A23187. It can also increase expression of neuronal Bcl2 protein, which may protect against apoptosis (30). GDNF is known to inhibit apoptosis and promote neuronal survival (7, 8, 25). GDNF can also rescue neonatal facial motor neurons and attenuate the lesion-induced decrease of choline acetyltransferase immunoreactivity in adult facial motor neurons after axotomy (48). However, the exact mechanism of the anti-apoptotic effect of GDNF is not known.

### *Nodal*

Recently a new class of TGF $\beta$ -like factors was identified on the basis of sequence similarity to nodal (14, 41). Mice with null mutations of nodal were found to lack appropriate notochord development (49). Subsequent studies established a role for nodal in the development of left-right asymmetry (9, 44). Many protein factors have multiple physiological properties throughout development and in adult animals. In the present study, we provide initial evaluation of nodal biology in rodent postnatal tissue. Immunoblot studies with oligopeptide antibodies to a carboxy terminal domain and to other domains suggest that nodal can be expressed in adult neural tissues (unpublished observations). This tissue distribution was confirmed by our preliminary PCR analysis of nodal RNA expression. More detailed stud-

ies of nodal cellular localization are under way. Although nodal appears to protect against excitotoxicity, high doses are required for this effect, and we suspect that other biological functions for nodal will ultimately be uncovered.

### CONCLUSIONS

Members of TGF $\beta$ -like trophic factors family, including GDNF, neurturin, persephin, nodal, and TGF $\beta$ 1, can protect motor neurons against chronic glutamate toxicity. Only GDNF and neurturin can induce motor axon outgrowth and this effect appears to be mediated through the RET receptor and the MAP kinase/PIK3 pathways. These compounds may be useful as potential therapeutic agents in treating neurodegenerative diseases such as ALS, as well as spinal cord/motor neuron and peripheral nerve regeneration.

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