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Nerve Growth Factor Induces Cell Cycle Arrest of Astrocytes

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Abstract

Neurotrophins can influence multiple cellular functions depending on the cellular context and the specific receptors they interact with. These neurotrophic factors have been extensively studied for their ability to support neuronal survival via Trk receptors, and to induce apoptosis via the p75^{NTR}. However, the p75^{NTR} is also detected on cell populations that do not undergo apoptosis in response to neurotrophins. In particular, we have detected p75^{NTR} expression on astrocytes during development and after seizure-induced injury. In this study we the role of NGF in regulating astrocyte proliferation, and in influencing specific aspects of the cell cycle. We demonstrate that NGF prevents the induction of cyclins and their association with specific cyclin-dependent kinases, and thereby prevents progression through the G1 phase of the cell cycle. Since we have previously shown that p75^{NTR}, but not TrkA, is expressed in astrocytes, these data suggest that activation of p75^{NTR} promotes withdrawal of astrocytes from the cell cycle, which may have important consequences during development and after injury.

Keywords

nerve growth factor; p75^{NTR}; cell cycle; proliferation; astrocyte

INTRODUCTION

Nerve growth factor (NGF) belongs to the neurotrophin family of growth factors (Levi-Montalcini and Angeletti, 1968) which also includes brain derived neurotrophic factor (BDNF) (Leibrock et al., 1989), neurotrophin 3 (NT3) (Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990) and neurotrophin 4/5 (NT4/5) (Berkemeier et al., 1991; Hallböök et al., 1991). NGF was initially identified for its role in growth, differentiation, and survival of sensory and sympathetic neurons during development and after injury (Levi-Montalcini, 1987), but now is known to influence many different cellular functions. NGF mediates its effects by binding to two structurally unrelated receptors, TrkA (Patapoutian and Reichardt, 2001; Chao, 2003; Huang and Reichardt, 2003) and the p75^{NTR}, which belongs to TNF receptor superfamily (Chao, 1994; Barker, 2004).

NGF is known to be produced by astrocytes under inflammatory conditions both in vivo (Oderfeld-Nowak and Bacia, 1994) and in vitro (Friedman et al., 1996). Moreover, the expression of p75^{NTR}, has been shown in astrocytes in vitro (Hutton et al., 1992; Semkova and Kriegelstein, 1999; Cragnolini et al., 2009) and in vivo (Rudge et al., 1994; Hanbury et al., 2002; Cragnolini et al., 2009), suggesting that astrocyte-produced NGF may have an

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autocrine or paracrine effect. We have recently demonstrated that NGF acting on p75^{NTR} attenuated proliferation induced by mitogens such as EGF or serum (Cragolini et al., 2009).

Regulation of the cell cycle depends on the coordinated production and interaction of two classes of regulatory proteins, cyclins and cyclin dependent kinases (cdks) (Sherr, 1993; Obaya and Sedivy, 2002). The activity of cdks is regulated by phosphorylation and by binding of inhibitory proteins called cyclin-dependent kinase inhibitors (CKI). Two families of cdk inhibitors delay or inhibit cell cycle progression. Ink4 proteins (p15, p16, p18 and p19) specifically target kinases cdk4 and cdk5 to prevent their binding with cyclin D (Serrano et al., 1993; Canepa et al., 2007). The CIP/KIP inhibitors (p21, p27 and p57) are more ubiquitous and associate with a broad range of cyclin-cdk complexes to inhibit their activity (Sherr and Roberts, 1995; Sherr, 1995; Sherr and Roberts, 1999).

NGF has been shown to induce (Zhang et al., 2003; Gigliozi et al., 2004; Lambert et al., 2004; Moser et al., 2004) or inhibit (Greene and Tischler, 1976; Ito et al., 2003; Evangelopoulos et al., 2004) proliferation depending on the cell type and the receptors expressed. Interestingly, the expression of NGF receptors changes cyclically during the cell cycle in PC12 cells (Urdiales et al., 1998), which may make the cells more or less responsive to NGF at different stages of the cell cycle.

The p75^{NTR} has been shown to regulate proliferation of many types of cancer cells, and is known as a tumor suppressor in a wide range of tumor cells affecting the machinery involved in cell cycle progression (Krygier and Djakiew, 2001; Krygier and Djakiew, 2002; Khwaja and Djakiew, 2003; Jin et al., 2007). The mechanisms connecting NGF and p75^{NTR} to the cell cycle are not completely understood. The p75^{NTR} lacks intrinsic catalytic activity thus its ability to signal depends on the interaction with intracellular proteins. To date several p75^{NTR} interacting molecules have been shown to be involved in regulation of the cell cycle. Schwann cell factor 1 (SC1) (Chittka and Chao, 1999; Chittka et al., 2004) and the receptor interacting protein 2 (RIP2) (Khursigara et al., 2001; Munz et al., 2002) inhibit proliferation, whereas the brain-expressed X-linked 1 (Bex1) protein competes with RIP2 for p75^{NTR} binding to maintain proliferation and inhibit differentiation (Vilar et al., 2006). Recently, a new p75^{NTR}-interacting protein associated with cell cycle arrest was identified, Sall2. This protein constitutively interacts with p75^{NTR} however NGF treatment causes a dissociation of Sall2 from p75^{NTR} and induces its translocation into the nucleus to facilitate cell cycle withdrawal and promote differentiation (Pincheira et al., 2009).

In this study we investigated the molecular mechanisms by which NGF and p75^{NTR} influence the progression of astrocytes through the cell cycle. We demonstrate that NGF interferes with several mechanisms responsible for the cell cycle progression, including inhibiting the synthesis of cyclins and their interaction with cdks, and preventing degradation of cdk inhibitors.

MATERIALS AND METHODS

Materials

Recombinant human NGF was generously provided by Genentech, and recombinant mouse EGF was purchased from Chemicon. Culture media was from Invitrogen, and poly-D-lysine, glucose, putrescine, progesterone, transferrin, selenium, insulin were purchased from Sigma. Antibodies to cyclin D1, p15^{INK4}, p27kip1, and cdk4 were from Cell Signaling Technologies, anti-ubiquitin was from Santa Cruz Biotechnology, anti-RRM1 was from Chemicon, and anti-cyclin E was from Abcam. Anti-GFAP was purchased from Roche, anti-p75^{NTR} was from Millipore, DAPI, methyl-scopolamine, pilocarpine hydrochloride, and

phenytoin were from Sigma, lactacystin was from Calbiochem, Diazepam was from Abbott Labs, and secondary antibodies were from Invitrogen.

Astrocyte cultures

Pregnant rats (Sprague-Dawley) were sacrificed by exposure to CO₂ and soaked in 80% ethanol for 10 minutes. Embryonic day 21 (E21) fetuses were removed under sterile conditions and kept in PBS on ice. Hippocampi were dissected, dissociated by trituration, and plated on poly-D-lysine-coated flasks in NM15 medium (Eagle's MEM with Earle's salts and 2mM L-glutamine, 15% heat-inactivated fetal bovine serum, 6mg/ml glucose, 0.5 µg/ml penicillin and 0.5 U/ml streptomycin). Astrocytes were grown to confluence and purified by differential shaking according to previously published methods (McCarthy and DeVellis, 1980; Cragolini et al., 2009). The astrocytes were trypsinized and replated at subconfluent density onto poly-D-lysine-coated dishes for Western blot analysis, or Lab-Tek slide chambers for immunocytochemistry. Astrocytes were plated in NM15 overnight, washed with PBS twice and changed to serum free medium consisting of a 1:1 mixture of Eagle's MEM and Ham's F12 supplemented with glucose (6 mg/mL), putrescine (60 µM), progesterone (20 nM), transferrin (100µg/mL), selenium (30 nM), penicillin (0.5 U/mL), and streptomycin (0.5 µg/mL). After 48 h of serum starvation, astrocytes were released from growth arrest by addition of EGF.

Immunoprecipitation and Western Blotting

Cells were washed with ice cooled PBS and harvested using RIPA buffer (50 mM Tris pH 7.8, 150mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.5% SDS, 5mM EDTA, protease inhibitor cocktail (Roche Products), 1mM sodium vanadate and 5mM sodium fluoride). Proteins were quantified using the Bradford assay (Bio-Rad) and equal amount of protein samples were run on SDS gels and transferred to nitrocellulose membrane. The blots were blocked in 5% non-fat dried skimmed milk in TBST for 1 hour. Then blots were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. The blots were washed with TBST, and incubated with secondary antibody for 1 hr at room temperature. The membranes were developed using either ECL (Pierce) or Odyssey infrared imaging system (LICOR Bioscience).

For p27kip1 ubiquitination studies, cells were treated with vehicle, EGF (10 ng/ml), NGF (100 ng/ml) or EGF+NGF for 12 hr, washed with PBS, lysed in buffer (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100, 5µM lactacystin and protease inhibitor cocktail and centrifuged at 14000 rpm for 15min at 4°C. Supernatants containing 200µg total protein in a final volume of 300µl were incubated with anti-cyclin E (1:200) overnight on a rocking platform at 4°C. Supernatants were incubated with protein A-agarose at 4°C for 2 hr. Immunoprecipitates were washed three times with lysis buffer and analyzed by Western blot for ubiquitin.

Immunocytochemistry

Serum starved astrocytes were treated with EGF, NGF or EGF+NGF for 12 hr. Cell were fixed with paraformaldehyde (4% in PBS) for 20 min, washed three times with PBS for 10 min, blocked in 10% goat serum/0.3% triton for 30 min, and incubated with anti-cyclin E (1:200) overnight at 4°C. Cells were then washed with PBS, and incubated with fluorescent secondary antibody for 1 hr in the dark at room temperature. After three washes with TBS, DAPI (1 µg/ml in PBS) was added and cells were visualized by fluorescence microscopy.

The quantification of cyclin E nuclei was performed as follows: three fields were counted per well. Two wells were used per treatment and each experiment was performed in triplicate. The results are expressed as a percentage of cyclin E positive cells/DAPI

normalized to control values. Statistical significance was determined by ANOVA followed by Newmann-Keuls post hoc analysis.

To analyze BrdU incorporation, astrocytes were grown to confluence in NM15 and the media was changed to SFM for 24 hr. Scratches were made on the surface of the dish with a yellow tip and the cells were exposed to vehicle, EGF (10 ng/ml), NGF (100 ng/ml), or EGF +NGF in the presence of BrdU (1 μ M). After 24 hr, the cells were fixed and stained for BrdU using the ABC kit (Vector Labs) with 3,3' diaminobenzidine (Sigma) as substrate. Positive astrocyte nuclei within and along the edges of the scratches were counted and are expressed as a percentage of the BrdU-positive astrocytes in control conditions.

Pilocarpine-induced seizures

Male Sprague-Dawley rats (250–275 g) were pre-treated for 0.5 hr with methyl-scopolamine (1 mg/kg, s.c.) and then treated with pilocarpine hydrochloride (350 mg/kg, i.p.). After 1 hr of status epilepticus, rats were treated with diazepam (10 mg/kg, i.p.) and phenytoin (50 mg/kg) to stop seizure activity. Additional diazepam was administered as necessary to prevent further seizures. Control animals received all the same treatments except they were injected with saline instead of pilocarpine. During recovery the animals were treated with Hartman's solution (130 mM NaCl, 4 mM KCl, 3 mM CaCl₂, 28 mM lactate; 1 ml/100 g) injected subcutaneously twice daily until the animals were capable of eating and drinking freely. Seven days later animals were anesthetized with ketamine hydrochloride (30 mg/kg) and xylazine (10 mg/kg) and perfused transcardially with saline followed by 4% paraformaldehyde. All animal studies were conducted using the NIH guidelines for the ethical treatment of animals with approval of the Rutgers Animal Care and Facilities Committee.

The brains were removed and postfixed in 4% paraformaldehyde for 1 hour, cryoprotected in 30% sucrose for 3 days, then snap frozen and kept at -80°C . Brain sections (12 μ m) were cut on a cryostat (Leica) and mounted onto charged slides. Sections were blocked in PBS/10% goat serum and permeabilized with PBS/0.3% Triton X-100, then exposed to anti-GFAP and anti-p75^{NTR} (07–476,) overnight at 4°C in PBS/0.3% triton. Slides were then washed 3X in PBS, exposed for 1 hr at room temp to secondary antibodies coupled to Alexa 488 and 594 fluorophores. Sections were coverslipped with anti-fading medium (ProLong Gold, Invitrogen, Oregon, USA) and analyzed by fluorescence microscopy (Nikon).

RESULTS

Kinetics of cyclin expression levels in EGF-stimulated astrocytes

We and others have previously shown that EGF stimulates DNA synthesis and proliferation of astrocytes (Huff and Schreier, 1989; Bramanti et al., 2007; Cragolini et al., 2009). Throughout the cell cycle the protein levels of the cyclins oscillate considerably, as a result of increased synthesis followed by degradation. To characterize the response of astrocytes to EGF we described the kinetics of cyclins D and E, which are key regulatory proteins that determine the progression of a cell through the G1-phase of the cell cycle. Astrocytes treated with EGF for the indicated times were lysed and subjected to Western blot analysis. Expression of cyclin D was clearly induced at 12 hr, remaining elevated until 24 hr, and started to decrease at 30 hr (figure 1). Basal levels of cyclin E expression were detected in untreated astrocytes however EGF treatment induced elevated cyclin E levels which were maximal between 18–24 hr, and reduced by 30 hr. Since the expression of cyclins D1 and E at 12 hr was significantly higher than control, we used this time point for further experiments.

NGF attenuates the induction of cyclins D1 and E

We have previously demonstrated that NGF causes a decrease in astrocyte cell number due to an inhibition of the progression through the S-phase of the cell cycle. To further investigate the role of NGF on the expression of cyclins D1 and E, cultured astrocytes were exposed to EGF in the presence or absence of NGF, cells were lysed and analyzed by Western blot. Immunoblot analysis showed that the presence of NGF with EGF attenuated the levels of cyclins D1 and E compared to astrocytes stimulated with EGF alone (figure 2A and B). Once induced, cyclins form complexes with cyclin-dependent kinases (cdks) and their activity is predominantly nuclear in location. Thus, the translocation of these cyclins to the nucleus is an indication that they are active. We used immunofluorescence to localize cyclin E in astrocytes treated with EGF alone, NGF alone, or with EGF + NGF. In the absence of EGF very little cyclin E was detected in either the cytoplasm or nucleus. However, after 12 hr of EGF treatment the percentage of the cells showing the presence of cyclin E in the nucleus was elevated to three times the control level. The presence of NGF significantly reduced the number of cyclin E positive nuclei compared to astrocytes treated with EGF alone, suggesting that NGF not only attenuated the expression of cyclin E but may also inhibit its translocation to the nucleus (figure 2C, D).

NGF inhibits the formation of cyclin D1-Cdk4 complexes induced by EGF

To assess whether NGF regulates other aspects of cyclin function, we next examined the association of cdk4 with cyclin D1, which is required for the progression from G1 into S phase. Astrocytes were treated with EGF, NGF, or both factors, and equal amounts of protein were immunoprecipitated with anti-cdk4 and probed for cyclin D1. EGF increased the amount of cyclin D1 associated with cdk4, and this increase was inhibited by the presence of NGF (figure 3). As expected, none of the treatments induced changes in the level of cdk4 protein, which does not change during the cell cycle.

NGF increases the expression of inhibitors associated with cdks

Cdk activity is regulated by phosphorylation and by binding of inhibitory proteins (CKIs). Two main classes of protein inhibitors bind to CDKs and inhibit their kinase activity. INK4 proteins, such as p15, target kinases such as cdk4 and cdk6 to prevent them from binding to cyclin D (Canepa et al., 2007). Another group of inhibitors, CIP/KIP, inhibits a broad range of cyclin-cdk complexes, including cdk2 (Besson et al., 2008; Vervoorts and Lüscher, 2008). Therefore we examined the regulation of two CKI proteins, p15INK4 and p27kip1, by NGF. Astrocytes were synchronized in serum-free media, treated for 12 hr with EGF, NGF, or both, and lysates were run in a Western blot and probed for p15INK or p27kip1. Basal levels of both inhibitors were detected in control conditions but they were both decreased by the presence of the mitogen EGF (figure 4). NGF reversed the effect of EGF on the expression of p15INK4. The expression of p27kip1 was slightly increased by NGF alone and the inhibition caused by EGF was partially reversed. These data suggest that there are several mechanisms by which NGF may restrain astrocyte cell cycle progression, including modulation of CKI expression.

NGF prevents ubiquitination of p27kip1 induced by EGF

The degradation of cyclins and their inhibitors is executed by the ubiquitin-proteasome system. The ubiquitin-dependent degradation of p27kip1 prevents it from blocking the cdk2-cyclin E complex allowing the cell cycle to progress (Montagnoli et al, 1999; Vervoorts and Lüscher, 2008). We investigated whether NGF was interfering with the proteasome-mediated degradation of p27kip1. Astrocytes were treated as indicated, and equal amounts of protein lysates were immunoprecipitated with anti-p27kip1, run in a Western blot and probed for ubiquitin. EGF treatment of astrocytes induced an accumulation of

polyubiquitinated p27kip1, which did not occur in the presence of NGF (figure 5). As shown in figures 4 and 5, levels of p27kip1 in lysates from astrocytes treated with EGF were low or undetectable, however the levels of polyubiquitinated p27kip1 were high. This discrepancy may be explained by the fact that the anti-p27kip1 antibody may not be capable of detecting the ubiquitinated form of the protein.

NGF attenuates BrdU incorporation after injury in culture

To assess the functional consequences of NGF treatment, we used a common in vitro injury model. Astrocytes were grown to confluence and changed to serum-free media. Scratches were made on the surface of the dish to simulate injury (figure 6A). BrdU was provided to the cultured astrocytes at the time of the scratch in the presence of vehicle, EGF, NGF, or EGF+NGF, and the number of astrocytes that incorporated BrdU along the edge of the scratch after 24 hr was evaluated (figure 6B). Significantly more BrdU-positive astrocytes were found along the edge of the scratches in the presence of EGF than in control. NGF alone had no effect on BrdU incorporation, however the presence of NGF together with EGF prevented the increase in BrdU-positive astrocytes seen with EGF alone, indicating that NGF prevented astrocytes from progressing through the G1 phase of the cell cycle after the scratch injury consistent with preventing the increases in cyclins D and E.

p75^{NTR} expression on astrocytes in vivo

The data presented here suggest that NGF may regulate astrocyte proliferation after an injury, at least in vitro. Interestingly, we have observed induction of p75^{NTR} on astrocytes in vivo in specific pathological situations, such as after pilocarpine-induced seizure (figure 7A). Moreover, specific sub-populations of astrocytes expressing a proliferation marker, RRM1 (Mann et al., 1988; Zhu et al., 2005), were detected after the seizure (figure 7B), suggesting that some astrocyte proliferation occurs in these conditions. One potential consequence of the induction of p75^{NTR} in astrocytes may be to regulate proliferation after an injury to attenuate gliosis and scar formation.

DISCUSSION

NGF influences multiple functions depending on the cellular context and the specific receptors expressed and activated. The effects of NGF on neurons are well characterized and range from maintaining survival and differentiation via TrkA, to triggering apoptosis via p75^{NTR} (Friedman, 2000). However, the effects on of NGF astrocytes have not been extensively characterized. We previously demonstrated that NGF causes an attenuation of astrocyte proliferation mediated by p75^{NTR} (Cagnolini et al., 2009). Although we have used NGF in these studies to activate p75^{NTR} on the astrocytes, it is not clear what the endogenous ligand might be that activates p75^{NTR} to regulate astrocyte proliferation in vivo. Since proNGF is a more potent and selective ligand for p75^{NTR}, this proneurotrophin may be the actual in vivo ligand. The goal of this study was to investigate the cellular mechanisms responsible for growth arrest of astrocytes induced by NGF. We demonstrate that NGF inhibits the synthesis of specific cyclins and their interaction with CDKs, and prevents degradation of specific CIKs.

The cyclic changes in the expression of cyclins and their association with cdks are indispensable for cell cycle progression in all multicellular eukaryotes (Satyanarayana and Kaldis, 2009). We observed that NGF attenuated the EGF-induced expression of cyclin D1, a key cyclin for the progression of the cell cycle through the G1/S phase. NGF treatment reduced, but did not completely prevent cyclin D and E induction by EGF, consistent with our previous study demonstrating that NGF attenuated, but did not completely inhibit, astrocyte proliferation (Cagnolini et al., 2009). Although NGF did not diminish Cdk4

levels, the association with cyclin D1 was greatly reduced. When astrocytes were treated with EGF we detected an increase in both cyclin E expression and accumulation in the nucleus, consistent with its role to target nuclear substrates. NGF attenuated the cyclin E expression and inhibited its translocation to the nucleus. These findings suggest that the inhibition of cyclin expression and the exclusion of cyclin E from the nucleus may be mechanisms that contribute to the inhibition of cell cycle progression by NGF.

The activities and functions of Cdk/cyclin complexes are regulated by two families of Cdk inhibitors, the INK4 family that bind to Cdk4 and Cdk6 and prevent D-type cyclin activity and the Cip/Kip family that inhibits Cdk2/cyclinE (Toyoshima and Hunter, 1994); (Aprelikova et al., 1995; O'Connor, 1997). The presence of NGF attenuated the degradation of p15INK and p27kip1, which is another mechanism by which NGF may arrest cell cycle progression. This is consistent with a previous study in neuroblastoma, in which an NGF-induced decrease in cell number was accompanied by an increase in p27kip1 levels (Woo et al., 2004). The increased levels of these CDK inhibitors following NGF treatment could result from insufficient capacity of the cell to degrade these proteins. The post-translational regulation of cell cycle proteins can be predominantly achieved by two types of protein modification, phosphorylation and ubiquitination. In astrocytes, inhibition of the ubiquitin-proteasome system by lactasystin has been shown to inhibit cell cycle progression and proliferation through modifying cell cycle related proteins (Ren et al., 2009). NGF affected the tightly controlled regulation of p27kip1 by inhibiting its ubiquitination. Other ubiquitinated proteins can also be positively or negatively influenced by NGF. For example, the binding of NGF to TrkA induces the internalization and ubiquitination of the receptor (Wooten and Geetha, 2006) and in PC12 cells NGF induces differentiation by repressing the ubiquitination of T-cadherin (Bai et al., 2007). In sympathetic neurons, NGF blocks the ubiquitin dependent degradation of Ret protein, increasing the levels of this protein and enhancing growth (Pierchala et al., 2007). These data suggest that NGF can affect the levels of certain proteins either by increasing or decreasing their catabolism, depending on the cell context.

We previously showed that NGF induces the expression of p75^{NTR} in astrocytes, which may result in differences in signaling in response to NGF throughout the cell cycle. The p75^{NTR} lacks intrinsic catalytic activity and signaling depends on the ability to recruit specific cytoplasmic proteins that interact with its intracellular domain and trigger different signaling pathways. Several p75^{NTR}-binding proteins have been identified that are implicated in regulating cell proliferation. In particular, SC1, acts as a transcriptional repressor of the promitotic gene, *cyclin E*, upon NGF treatment and thus blocks DNA replication (Chittka et al., 2004). Interestingly, a recent publication demonstrated that the NGF promotes the association of the p75^{NTR} intracellular domain with the cyclin E promoter region and can modulate cyclin E1 levels in PC12 cells (Parkhurst et al., 2010). Additional p75^{NTR}-interacting proteins such as RIP2, Bex1, and Sall2, have also been implicated as regulators of the cell cycle (Khursigara et al. 2001; Vilar et al, 2006; Pincheira et al., 2009).

The cell cycle arrest induced by NGF has been associated with the initiation of differentiation. PC12 cells respond to NGF by differentiating, and cells accumulate in the G1 phase of the cell cycle (van Grunsven et al., 1996). In C6 astrocytoma cells NGF inhibited proliferation and induced morphological changes, including the formation of growth cones, outgrowth of processes and cellular hypertrophy, indicating that exogenous NGF stimulated differentiation and inhibited proliferation of these cells (Watanabe et al., 1999).

The precise biological significance of the growth arrest and its role in reactive astrogliosis has not been elucidated yet, however it might have several consequences for nervous system

function, especially after an injury. Consistent with the possibility that NGF modulates aspects of gliosis, Cirillo and colleagues (2009) showed that NGF reduced the reactive astrogliosis associated with neuropathic pain. NGF also was able to restore glial and neuronal amino acid transporters (Cirillo et al., 2011). The potential outcome of NGF treatment on astrocytes may depend on the neurotrophin receptors expressed in a particular cellular context. Our in vitro experiments demonstrated that a scratch lesion increased levels of p75^{NTR} in the astrocytes, and that NGF prevented the EGF-induced BrdU incorporation in astrocytes after a scratch lesion, consistent with the idea that activation of p75^{NTR} may serve to attenuate gliosis after injury. In vivo, we have observed that p75^{NTR} is induced on astrocytes after seizures, although the specific role mediated by p75^{NTR} under these conditions remains unclear. Moreover, although we have used NGF to activate p75^{NTR}-mediated cell cycle arrest in these studies, many ligands can interact with this receptor, and although NGF levels are induced after injury, the endogenous ligand that may elicit these responses has not been elucidated.

Altogether our results reveal that profound changes in the cell cycle regulatory machinery can be mediated by the p75^{NTR}, which signals astrocytes to withdraw from the cell cycle in response to NGF. During development, activation of p75^{NTR} may participate in a regulatory mechanism for the cessation of proliferation prior to differentiation, and after injury the induction of p75^{NTR} may serve to attenuate the extent of gliosis. The possibility that this neurotrophin receptor may regulate reactive gliosis in vivo after an injury or a neurodegenerative disease requires further investigation.

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Figure 1.

Kinetics of cyclin D1 and E expression levels in EGF treated astrocytes. Serum starved astrocytes were treated with 10ng/ml EGF for 6, 12, 18, 24 or 30 hr and subjected to Western blot analysis. Representative immunoblots show induction of cyclins D1 and E. Blots were stripped and reprobed for β -actin as a loading control. Results are representative of three independent experiments.

Figure 2.

NGF decreases the expression of cyclin D1 and E induced by EGF. Synchronized astrocytes were incubated for 12 hr in SFM alone or containing EGF (10ng/ml), NGF (100ng/ml) or both. Cells were lysed and lysates subjected to Western blot analysis. The increased expression of both cyclin D1 (A) and cyclin E (B) induced by EGF were attenuated by NGF. Blots were stripped and reprobed for β -actin as a loading control. Data from 3 independent experiments were quantified and shown in the graphs below the blots. C) Astrocytes were treated for 12 hr, fixed and immunostained for Cyclin E (red) and DAPI (blue) to localize nuclei. Note the abundance of pink nuclei indicating the overlay of cyclin E immunostaining with DAPI in EGF treatment. The translocation was quantified as the ratio of cyclin E

positive cells to DAPI positive cells (D). Asterisks in A, B, and D indicate $p < 0.05$ by ANOVA followed by Newmann-Keuls post hoc analysis.

Figure 3.

NGF inhibits the interaction of cyclin D1 with cdk4 induced by EGF. Synchronized astrocytes were incubated in SFM alone or containing EGF (10ng/ml), NGF (100ng/ml) or both for 12h. Cdk4 was immunoprecipitated from equal amounts of protein. The immunoprecipitates were separated by SDS-PAGE and probed for cyclin D1. Blots were re-probed for cdk4 (bottom). Quantification indicates the ratio of Cyclin D1 to cdk4 from three independent experiments. Asterisk indicates $p < 0.05$ by ANOVA followed by Newmann-Keuls post hoc analysis.

Figure 4.

NGF reverses the inhibition of p15INK4 and p27kip1 by EGF. Synchronized astrocytes were incubated in SFM alone or containing EGF (10ng/ml), NGF (100ng/ml) or both for 12h. Representative immunoblot showing the expression of p15INK4 and p27kip1 are shown. The treatment with NGF prevented the decrease of p15INK4 and attenuated the decrease of p27kip1 caused by EGF. Blots were stripped and reprobed for β -actin as a loading control. Graphs show quantification of results from three independent experiments. Asterisk indicates different from other groups at $p < 0.05$ by ANOVA followed by Newmann-Keuls post hoc analysis.

Figure 5.

NGF prevents ubiquitin-mediated degradation of p27kip1. Hippocampal astrocytes were treated for 12h with SFM, NGF (100 ng/ml) EGF (10ng/ml) or both. The lysates were immunoprecipitated using anti-p27kip1, separated by SDS-PAGE and blots probed with anti-ubiquitin (Ubq). Lysates from the same samples used for immunoprecipitation were subjected to Western blot analysis and probed for p27kip1 (bottom). Results are representative of three independent experiments.

Figure 6.

NGF attenuates BrdU incorporation after scratch injury in vitro. A) Image showing the incorporation of BrdU into nuclei of astrocytes along the edges of the scratch, indicated by arrows. B) Western blot showing increased p75NTR expression in the astrocytes 6 hr following the scratch lesions. Blot was striped and re-probed for actin as a loading control. C) Quantification of astrocytes incorporating BrdU after the scratch lesion in the presence of EGF, NGF, or EGF+NGF and expressed as percent control. Asterisk indicates significant at $p < 0.05$ by ANOVA with Neumann Keuls post-hoc analysis, $n=6$.

Figure 7. p75^{NTR} expression on astrocytes in vivo. A) By three days after pilocarpine-induced seizures, p75^{NTR} was detected on numerous astrocytes in the CA3 region of the hippocampus. B) Double-labeling for GFAP and RRM1 demonstrating the presence of proliferating astrocytes in the hippocampus after the seizure. Size bar equals 50 μ m.