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, the authors have detected p75^{NTR} expression on astrocytes during development and after seizure-induced injury. In this study, the authors investigated the role of Nerve growth factor (NGF) in regulating astrocyte proliferation and in influencing specific aspects of the cell cycle. The authors have demonstrated 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 the authors 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. © 2011 Wiley Periodicals, Inc. Develop Neurobiol 72: 766–776, 2012

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 (Leibrock et al., 1989), neurotrophin 3 (Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990), and neurotrohin 4/5 (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 Krieglstein, 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 autocrine or paracrine effect. The authors have recently demonstrated that NGF acting on p75^{NTR} attenuated proliferation induced by mitogens such as EGF or serum (Cragnolini et al., 2009).

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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; Gigliozzi 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, 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, the authors have investigated the molecular mechanisms by which NGF and $p75^{NTR}$ influence the progression of astrocytes through the cell cycle. The authors have demonstrated 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 killed by exposure to CO_2 and soaked in 80% ethanol for 10 min. 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 2 m*M* L-glutamine, 15% heat-inactivated fetal bovine serum, 6 mg/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; Cragnolini 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 n*M*), transferrin (100 μg/mL), selenium (30 n*M*), 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 m*M* Tris pH 7.8, 150 m*M* NaCl, 1% NP40, 0.5% deoxycholic acid, 0.5% SDS, 5 m*M* EDTA, protease inhibi-

tor cocktail (Roche Products), 1 m*M* sodium vanadate and 5 m*M* 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% nonfat-dried-skimmed milk in TBST for 1 h. 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 h 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 h, washed with PBS, lysed in buffer (50 m*M* Tris/HCl (pH 7.5), 150 m*M* NaCl, 1 m*M* EDTA, 5 m*M* NaF, 1 m*M* Na_3VO_4, 1% Triton X-100, 5 μM lactacystin, and protease inhibitor cocktail) and centrifuged at 14,000 rpm for 15 min at 4°C. Supernatants containing 200 μg total protein in a final volume of 300 μL 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 h. Immunoprecipitates were washed thrice with lysis buffer and analyzed by Western blot for ubiquitin.

Immunocytochemistry. Serum-starved astrocytes were treated with EGF, NGF, or EGF + NGF for 12 h. Cell were fixed with paraformaldehyde (4% in PBS) for 20 min, washed thrice 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 h 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 Newman–Keuls *post hoc* analysis.

To analyze BrdU incorporation, astrocytes were grown to confluence in NM15 and the media was changed to SFM for 24 h. 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 μM). After 24 h, 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 pretreated for 0.5 h with methyl-scopolamine (1 mg/kg, s.c.) and then treated with pilocarpine hydrochloride (350 mg/kg, i.p.). After 1 h 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 m*M* NaCl, 4 m*M* KCl, 3 m*M* CaCl, 28 m*M* 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 h, cryoprotected in 30% sucrose for 3 days, then snap frozen and kept at −80°C. Brain sections (12 μm) 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 thrice with PBS, exposed for 1 h at room temperature to secondary antibodies coupled to Alexa 488 and 594 fluorophores. Sections were coverslipped with anti-fading medium (ProLong Gold, Invitrogen, Oregon) and analyzed by fluorescence microscopy (Nikon).

RESULTS

Kinetics of Cyclin Expression Levels in EGF-Stimulated Astrocytes

The authors and others have previously shown that EGF stimulates DNA synthesis and proliferation of astrocytes (Huff and Schreier, 1989; Bramanti et al., 2007; Cragnolini 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, the authors have 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 h, remaining elevated until 24 h, and started to decrease at 30 h (Fig. 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 and 24 h, and reduced by 30 h. Since the expression of cyclins D1 and E at 12 h was significantly higher than control, the authors used this time point for further experiments.

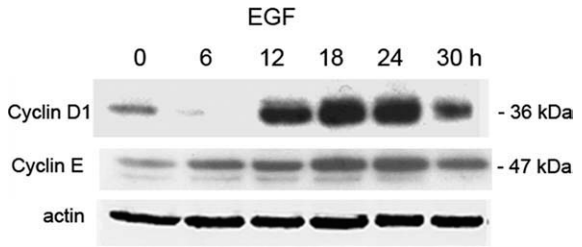

Figure 1 Kinetics of cyclin D1 and E expression levels in EGF-treated astrocytes. Serum-starved astrocytes were treated with 10 ng/mL EGF for 6, 12, 18, 24, or 30 h and subjected to Western blot analysis. Representative immunoblots show induction of cyclins Dl and E. Blots were stripped and reprobed for β-actin as a loading control. Results are representative of three independent experiments.

NGF Attenuates the Induction of Cyclins D1 and E

The authors 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 with astrocytes stimulated with EGF alone [Fig. 2(A,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. The authors 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 h of EGF treatment, the percentage of the cells showing the presence of cyclin E in the nucleus was elevated thrice the control level. The presence of NGF significantly reduced the number of cyclin E positive nuclei when compared with 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 [Fig. 2(C,D)].

NGF Inhibits the Formation of Cyclin D1–Cdk4 Complexes Induced by EGF

To assess whether NGF regulates other aspects of cyclin function, the authors have 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

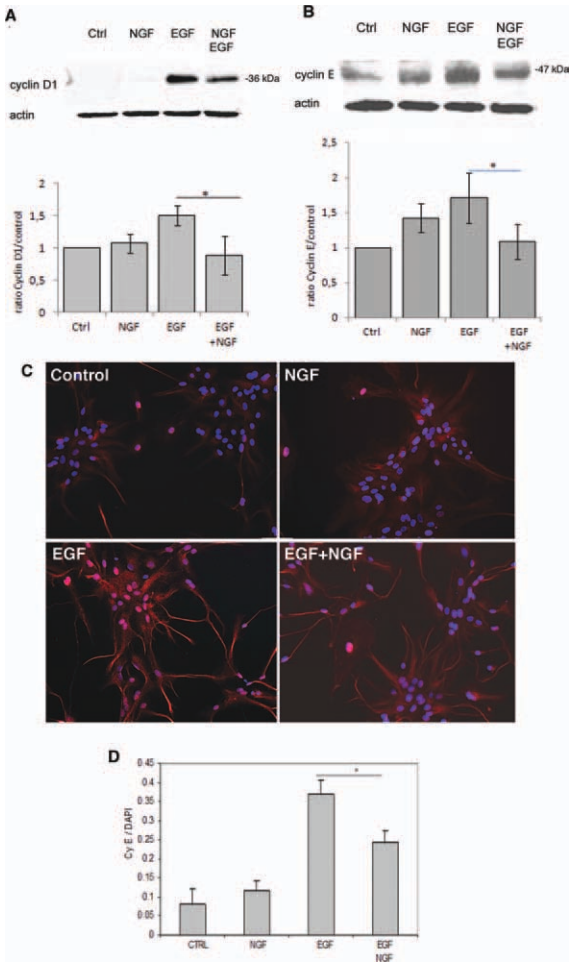

Figure 2 NGF decreases the expression of cyclin D1 and E induced by EGF. Synchronized astrocytes were incubated for 12 h in SFM alone or containing EGF (10 ng/mL), NGF (100 ng/mL), or both. Cells were lysed and lysates subjected to Western blot analysis. The increased expression of both cyclin D1 (A) 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 three independent experiments were quantified and shown in the graphs below the blots. (C) Astrocytes were treated for 12 h, 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 Newman–Keuls *post hoc* analysis.

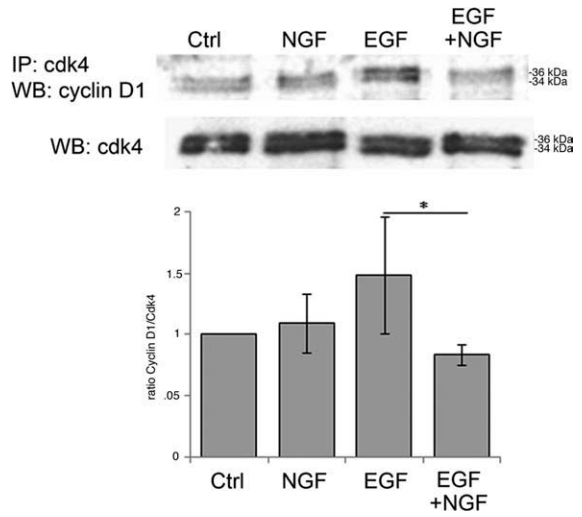

Figure 3 NGF inhibits the interaction of cyclin D1 with cdk4 induced by EGF. Synchronized astrocytes were incubated in SFM alone or containing EGF (10 ng/mL), NGF (100 ng/mL) or both for 12 h. Cdk4 was immunoprecipitated from equal amounts of protein. The immunoprecipitates were separated by SDS-PAGE and probed for cyclin D1. Blots were reprobed 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 Newman–Keuls *post hoc* analysis.

increased the amount of cyclin D1 associated with cdk4, and this increase was inhibited by the presence of NGF (Fig. 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 (CIKs). 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 Luscher, 2008). Therefore, the authors have examined the regulation of two CIK proteins, p15INK4 and p27kip1, by NGF. Astrocytes were synchronized in serum-free media, treated for 12 h 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 (Fig. 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 ubiq-

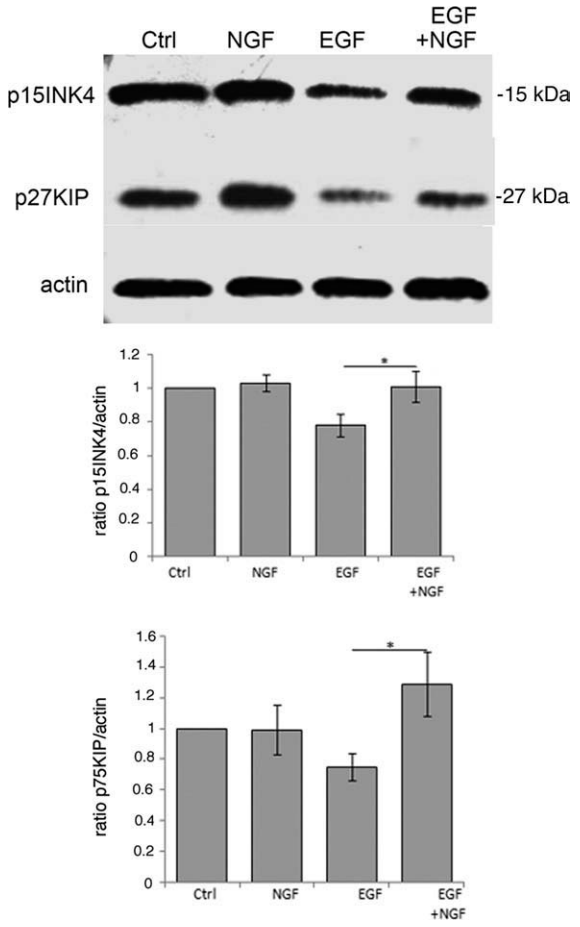

Figure 4 NGF reverses the inhibition of p15INK4 and p27kip1 by EGF. Synchronized astrocytes were incubated in SFM alone or containing EGF (10 ng/mL), NGF (100 ng/mL), or both for 12 h. Representative immunoblot showing the expression of p15INK4 and p27kip1 are shown. The treatment with NGF prevented 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 Newman–Keuls *post hoc* analysis.

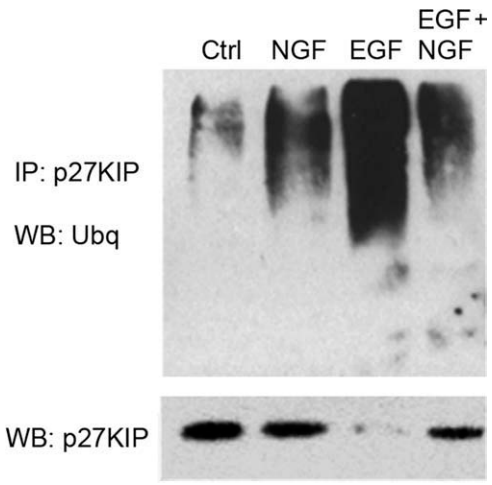

Figure 5 NGF prevents ubiquitin-mediated degradation of p27kip1. Hippocampal astrocytes were treated for 12 h with SFM, NGF (100 ng/mL) EGF (10 ng/mL), or both. The lysates were immunoprecipitated using anti-p27kip1, separated by SDS-PAGE and blots probed with anti-ubiquitin (Ubq). Lysates from the same samples used for immunoprecipitation were subjected to Western blot analysis and probed for p27kip1 (bottom). Results are representative of three independent experiments.

uitin-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). The authors have 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 (Fig. 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, the authors have 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 [Fig. 6(A)] resulting in increased levels of p75 [Fig. 6(B)]. 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 h was evaluated [Fig. 6(C)]. 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 in this article suggest that NGF may regulate astrocyte proliferation after an injury, at

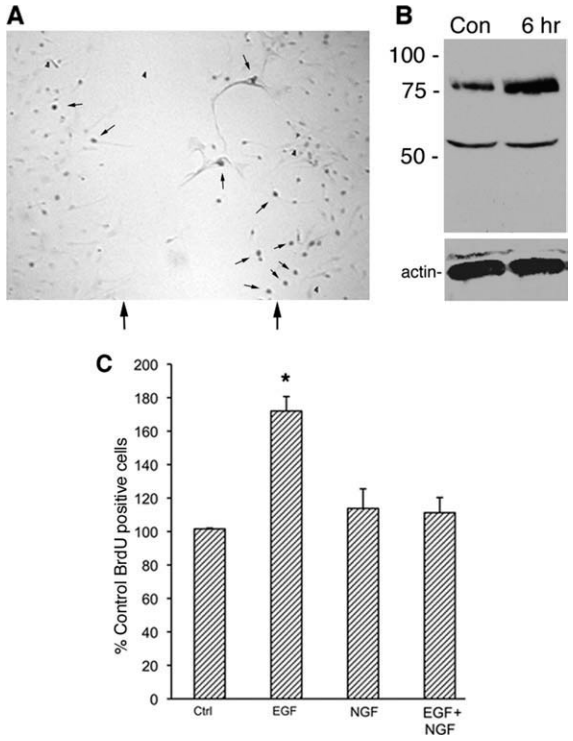

Figure 6 NGF attenuates BrdU incorporation after scratch injury *in vitro*. (A) Image showing the incorporation of BrdU into nuclei of astrocytes along the edges of the scratch, indicated by arrows. (B) Western blot showing increased p75NTR expression in the astrocytes 6 h following the scratch lesions. Blot was striped and reprobed 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 Neuman–Keuls *post hoc* analysis, $n = 6$.

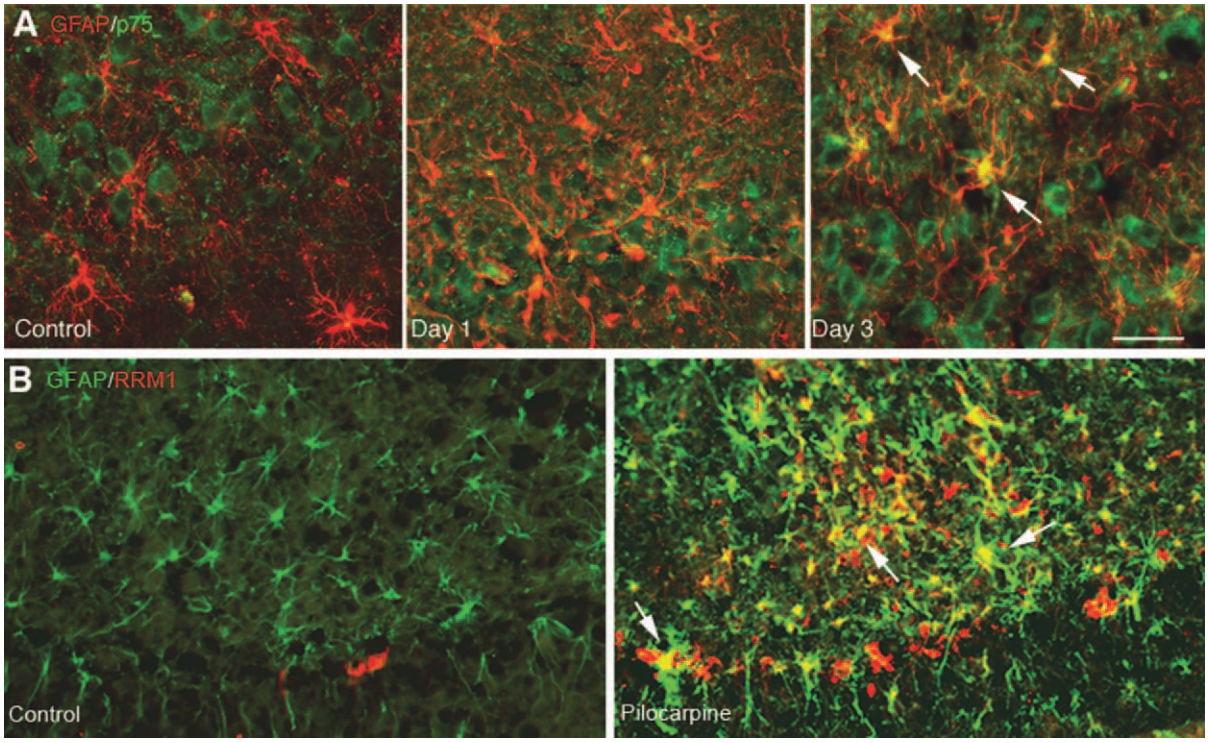

Figure 7 $p75^{NTR}$ expression on astryocytes *in vivo*. (A) By 3 days after pilocarpine-induced seizures, $p75^{NTR}$ was detected on numerous astrocytes in the CA3 region of the hippocampus. (B) Double-labeling for GFAP and RRM1 demonstrating the presence of proliferating astrocytes in the hippocampus after the seizure. Size bar = 50 μm.

least *in vitro*. Interestingly, the authors have observed induction of $p75^{NTR}$ on astrocytes *in vivo* in specific pathological situations, such as after pilocarpine-induced seizure [Fig. 7(A)]. Moreover, specific subpopulations of astrocytes expressing a proliferation marker, RRM1 (Mann et al., 1988; Zhu et al., 2005), were detected after the seizure [Fig. 7(B)], 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. The authors have previously demonstrated that NGF causes an attenuation of astrocyte proliferation mediated by $p75^{NTR}$ (Cragnolini et al., 2009). Although, the authors 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. The authors have demonstrated 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). The authors 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

(Cragnolini et al., 2009). Although NGF did not diminish Cdk4 levels, the association with cyclin D1 was greatly reduced. When astrocytes were treated with EGF, the authors have 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.

The authors 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 cylin 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 et al. (2010) showed that NGF reduced the reactive astrocytosis 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*, the authors 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 the authors 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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