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p75^{NTR} Expression Is Induced in Isolated Neurons of the Penumbra After Ischemia by Cortical Devascularization

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The p75 neurotrophin receptor (p75^{NTR}) is involved in neuronal functions ranging from induction of apoptosis and growth inhibition to the promotion of survival. p75^{NTR} expression is induced in the central nervous system (CNS) by a range of pathological conditions, where it seems to have a role in neuronal death and axonal growth inhibition. The cellular mechanisms driving p75^{NTR} expression in cell lines and primary neurons is Sp1 dependent (Ramos et al. [2007] J. Neurosci. 27:1498). In this study, we analyzed the spatiotemporal profile of p75^{NTR} expression after an ischemic lesion induced by cortical devascularization (CD). Our results show that p75^{NTR} expression occurs in isolated neurons of the ischemic lesion site. The p75^{NTR+} neurons presented morphological alterations and active caspase-3 staining. Some p75^{NTR+} neurons were also positive for sortilin. The peak of p75^{NTR} expression was localized 3 days postlesion (3DPL) in the penumbra. Sp1 transcription factor nuclear localization was observed in p75^{NTR+} neurons. The overall level of Sp1 expression was increased until 14DPL on the ipsilateral hemisphere. With primary cortical neurons, we demonstrated that p75^{NTR} expression is induced by excitotoxic stress and correlated with increased Sp1 abundance. We conclude that p75^{NTR} expression is localized in selected neurons of the ischemic lesion and that these neurons are probably condemned to apoptotic cell death. In primary neuronal culture, it is clear that excitotoxity and Sp1 are involved in induction of p75^{NTR} expression, although, in vivo, some additional mechanisms are likely to be involved in the control of p75^{NTR} expression in specific neurons in vivo.

Key words: neurotrophin; ischemia; Sp1; glutamate; neuronal death; sortilin

Neurotrophins are a family of soluble ligands playing critical roles in development and maintenance of the nervous system. Initially characterized for their ability to promote the survival of peripheral neurons, neurotrophins are now known to play other important physiological roles that include activating apoptosis, regulating growth, and modulating synaptic function (Kaplan and Miller, 2000; Bibel and Barde, 2000; Liebl et al., 2001). This functional diversity results from neurotrophin binding to the p75 neurotrophin receptor (p75^{NTR}) and to the Trk receptors. In the last several years, p75^{NTR} has emerged as a key player in the regulation of neuronal cell death and regeneration by interacting with mature neurotrophins and immature proneurotrophins (Roux and Barker, 2002; Barker, 2004).

p75^{NTR} is highly expressed during development, but, in most adult tissues, including the brain, p75^{NTR} is maintained at low levels (Roux and Barker, 2002). However, p75^{NTR} levels rise dramatically after injury to the peripheral or central nervous system (CNS). For example, in rats subjected to pilocarpine-induced seizures, neuronal p75^{NTR} transcription is dramatically increased in cortical and hippocampal neurons (Roux et al., 1999). Importantly, there is a tight correlation between induced p75^{NTR} expression and subsequent apoptosis of central neurons in animal models of epileptic seizures (Roux et al., 1999; Troy et al., 2002). Animals

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rendered null for p75^{NTR} show a dramatic reduction of injury-induced apoptosis, demonstrating a key role for p75^{NTR} in this form of cell loss. p75^{NTR} is also induced in the periphery, most notably in distal segments of peripheral nerves subjected to lesion or crush, where it seems to contribute to apoptosis and growth inhibition (Ferri and Bisby, 1999; Bentley and Lee, 2000).

Induction of p75^{NTR} expression in ischemia has been demonstrated to occur in transient global ischemia in hippocampal astrocytes (Soltys et al., 2003; Oderfeld-Nowak et al., 2003) and in striatal cholinergic neurons that survive the insult (Andsberg et al., 2001; Greferath et al., 2002). Focal ischemia also induces p75^{NTR} expression in apoptotic neurons in gerbil hippocampus (Bagum et al., 2001). Recently, the proneurotrophins have emerged as proapoptotic ligands (mainly pro-NGF and pro-BNDF) that bind a receptor complex consisting of p75^{NTR} and sortilin (Harrington et al., 2004; Barker, 2004). Proneurotrophins are released from injured CNS and seem to interact with the p75^{NTR}–sortilin complex to induce apoptotic neuronal death (Harrington et al., 2004). Also, ageing and Alzheimer's disease are known to increase proneurotrophins level in the CNS (Bierl and Isaacson, 2007; Cuello and Bruno, 2007), reflecting the importance of p75^{NTR} expression as a final control point to allow neuronal death after injury.

The control of p75^{NTR} expression is starting to be understood. In vitro studies have demonstrated that increased level of Sp1 transcription factor and its binding to the proximal p75^{NTR} promoter are essential to induce p75^{NTR} expression in neurons subjected to hypoosmotic stress (Ramos et al., 2007). Because p75^{NTR} proapoptotic ligands and intracellular adaptors are available after brain injury (Sarret et al., 2003; Harrington et al., 2004; Barker, 2004), the p75^{NTR} expression emerges as the last control point for induction of neuronal apoptosis.

In this report, we analyze the spatiotemporal profile of p75^{NTR} expression in a model of brain ischemia and study in cortical primary neurons the excitotoxicity potential involvement in induction of p75^{NTR} expression. Our results show that p75^{NTR} is expressed in isolated neurons of the ischemic penumbra that present morphological characteristics of degenerating neurons, sortilin expression, and active caspase-3 staining. In primary cortical neurons, excitotoxicity by glutamate exposure effectively induced p75^{NTR} expression and increased Sp1 abundance.

MATERIALS AND METHODS

Reagents

Cell culture reagents were obtained from Hyclone (Logan, UT) and Invitrogen (Carlsbad, CA). Antibodies were purchased from Upstate Biotechnology (Lake Placid, NY; anti-Sp1, catalog No. 07-645; anti-p75^{NTR}, catalog No. 07-476); from Alomone (Jerusalem, Israel; antisortilin, catalog No. ANT-009); from Oncogene (Cambridge, MA; anti-p75^{NTR} clone MC192); from Chemicon (Temecula, CA; cleaved caspase-3, catalog No. AB3623; NeuN, catalog No. MAB377). The horseradish peroxidase-conjugated secondary antibodies, biotinylated antibodies, extravidin complex, glutamate, and other chemicals were obtained from Sigma (St. Louis, MO). Secondary fluorescent antibodies (Fab2 fragment) were from Immunotech Coulter (Fullerton, CA). The enhanced chemiluminescence (ECL) used in the detection of immunoreactive bands was purchased from Pierce (Rockford, IL).

Cortical Devascularization

Adult male Wistar rats (250–300 g) obtained from the Animal Facility of the Pharmacy and Biochemistry School, University of Buenos Aires, were used in this study. Animals were housed in a controlled environment (12/12-hr light/dark cycle, controlled humidity and temperature, free access to standard laboratory rat food and water). All surgical procedures and perfusions were performed with animal's under gas anesthesia induced with sevofluorane (4% vol/vol). Animals were subjected to a unilateral cortical devascularization (CD) lesion as previously described (Herrera and Robertson, 1989; Figueiredo et al., 1993; Ramos et al., 2004). Briefly, rats were placed in a stereotaxic apparatus, and a small surface of skull between the coronal suture and the bregma line was removed to expose the underlying vasculature. A 27-gauge needle was used to cut the overlying dura and tear it away from the underlying pia. A sterile cotton swab was then used to tear back the pia and to disrupt the pial blood vessels overlaying the exposed cortex. Immediately, small sterile cotton pieces embedded in sterile saline solution were laid on the cortical surface until all bleeding ceased (usually less than 50 sec). All cotton pieces were removed, and the incision in the overlying skin was then closed using the temporal muscle and the attached fascia to cover the lesion site. The animals were placed in separate cages to allow them to recover after the surgery for 1, 3, 7, and 14 days. Sham animals were prepared by removing the skull as indicated, leaving the vasculature intact. During the surgery and the whole awakening period, animal temperature was maintained by means of a heating pad. The rectal temperature of animals was monitored to preclude hypothermia and to maintain 37.5°C ± 0.5°C until animals were awake. The animal care for this experimental protocol was in accordance with the NIH guidelines for the care and use of laboratory animals and the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience.

Fixation

After various recovery times [1, 3, 7, or 14 days postlesion (DPL)], animals were deeply anesthetized with 300 mg/kg chloral hydrate (i.p.) and were perfused through the left ventricle, initially with saline solution with 5,000 UI/ml heparin and subsequently with a fixative solution containing 4% w/v paraformaldehyde and 0.25% w/v glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Subsequent to delivery of 300 ml of fixative solution through a peristaltic pump, brains were removed and kept in the cold fixative solution for 90 min. Brains were washed three times in cold 0.1 M phosphate buffer, pH 7.4, containing 5% w/v sucrose and left in a

cryoprotective solution (0.1 M phosphate buffer plus 30% w/v sucrose) for 48 hr at 4°C. Then, brains were snap frozen, and coronal 25–50-μm-thick brain sections were cut with a cryostat. Free-floating sections were kept in a cryoprotective solution (30% glycerol, 20% ethylene glycol in 0.05 M phosphate buffer) at −20°C until use.

Immunohistochemistry

Brain sections of animals belonging to the different experimental groups were simultaneously processed in the free-floating state as previously described (Ramos et al., 2000, 2004). Briefly, sections to be used for light microscopy were dehydrated, and endogenous peroxidase activity was inhibited with 0.5% v/v H_2O_2 in methanol for 30 min at room temperature. After rehydration, brain sections were blocked for 1 hr with 3% v/v normal goat serum (NGS) in phosphate-buffered saline (PBS). After rinses in PBS, sections were incubated for 48 hr at 4°C with the primary antibodies and then rinsed and incubated for 1 hr at room temperature with biotinylated secondary antibodies diluted 1:200. After further washing in PBS, sections were incubated for 1 hr with the Extravidin complex solution diluted 1:400. After washing again five times in PBS and twice in 0.1 M acetate buffer, pH 6, development of peroxidase activity was carried out with 0.035% w/v 3,3-diaminobenzidine plus 2.5% w/v nickel ammonium sulfate and 0.1% v/v H_2O_2 dissolved in acetate buffer. After the enzymatic incubation step, sections were washed with distilled water and mounted on gelatin-coated slides, dehydrated, and coverslipped with Permount. For double-immunofluorescence studies, peroxidase inhibition was omitted, nonspecific binding in tissue sections was blocked with 10% NGS, the secondary antibodies (Fab2 labeled with FITC or rhodamine; Immunotech-Coulter) were used at 1:400, and sections were mounted with glycerol 30% in PBS. All antibodies, as well as Extravidin complex, were dissolved in PBS containing 1% v/v NGS and 0.3% v/v Triton X-100, pH 7.4. The dilutions of the primary antibodies were 1/1,000 $p75^{NTR}$ or $p75^{NTR}$ MC192, 1/500 active caspase-3, 1/1,000 NeuN, 1/1,000 sortilin, 1/1,000 Sp1. Controls for the immunohistochemistry procedure were routinely performed by omitting the primary antibody. These control sections did not develop any immunohistochemical labeling. Photographs were taken with a Zeiss Axiophot microscope equipped with an Olympus Q-Color 5 cooled camera.

Hoechst 33342 Staining

Brain sections of animals exposed to the different recovery times were simultaneously processed in a free-floating state. After the single or double immunohistochemistry as detailed above, sections were rinsed with PBS three times and incubated with a solution of Hoechst 33342 (2 μg/ml in PBS), in a dark chamber, for 5 min at room temperature. After further rinses in PBS, the sections were mounted on gelatin-coated slides, coverslipped with a solution of glycerol 30% in PBS, and photographed as described above.

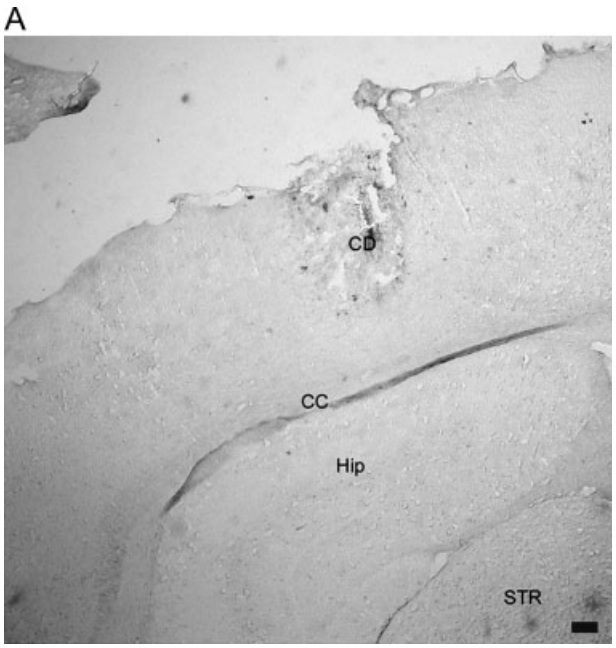


Fig. 1. **A:** The cortical devascularizing ischemic lesion affects brain cortical layers I–IV. The photograph shows a typical CD lesion at 3DPL. CD, cortical devascularizing lesion; CC, corpus callosum; Hip, hippocampus; STR, striatum. **B:** The appearance of ischemic core and penumbra in the cortical devascularizing lesion. A: The presence of $p75^{NTR}$-positive neurons (arrowheads) can be visualized in the penumbra surrounding the ischemic core at 3DPL; thick arrows show the limit of ischemic core, and thin arrows show the penumbra limits. B: p75-Immunostained cells. C: Nuclear Hoechst staining showing a multitude of cells in the area of the ischemic lesion. D: Merged image of $p75^{NTR}$ and Hoechst staining showing that only a small portion of cells is $p75^{NTR}$ positive. **C:** The morphology of the $p75^{NTR}$-immunoreactive neurons showed features of neuronal degeneration. Neurons stained with an anti-$p75^{NTR}$ antibody that recognizes the intracellular domain (ICD) at 3DPL. A,B: Visualization of the ischemic lesion induced by the cortical devascularization paradigm in two different animals to show the constant aspect of the lesion and the distribution of $p75^{NTR}$-positive neurons. C–G: Different images of $p75^{NTR}$ positive neurons in the ischemic core showing characteristics of degenerating neurons. H: Isolated fibers labeled with $p75^{NTR}$ in the penumbra. I: High-power photograph of degenerating $p75^{NTR}$ positive neurons. **D:** Details of nuclear morphology of $p75^{NTR}$-immunoreactive cells. Cells were labeled with anti-$p75^{NTR}$ antibody that recognizes the intracellular domain and stained with the nuclear staining Hoechst 33342. Note chromatin condensation and other morphological features indicative of a process of cell degeneration (shorter projections, irregular soma, diffuse nuclear-cytoplasm limits and absence of nucleolus). A large number of $p75^{NTR}$ negative cells are present in the surrounding area and show normal and altered nucleus. Scale bars = 100 μm in A,CA,CB; 50 μm in B,CC,CD; 25 μm in CE–CH; 10 μm in CI and D. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Primary Neuronal Culture

The cortical neuronal cultures were prepared from embryonic day (E) 16 Wistar rats according to Goslin et al. (1988), with modifications. Briefly, hippocampal neurons were dissociated by treatment with 0.25% trypsin, followed by

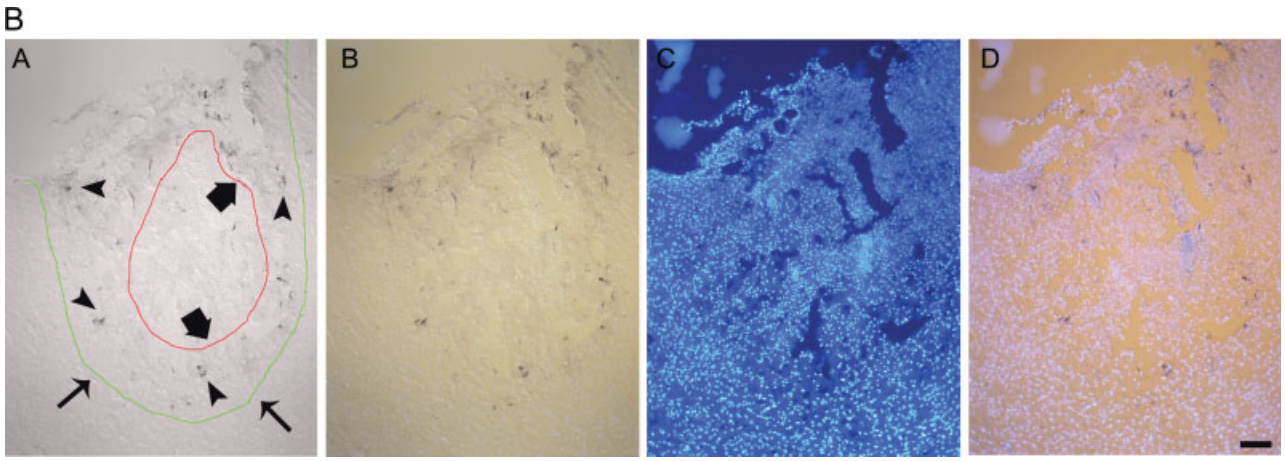


Figure 1. (Continued)

trituration through a Pasteur pipette. Cells plated at a density of 1×10^5 cells/cm^2 or 7×10^3 cells/cm^2 on poly-D-lysine-coated plastic dishes or glass coverlips, respectively, and maintained for up to 18 days in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen) and 0.5 mM glutamine. For immunocytochemistry, neurons were washed with cold PBS and fixed with 4% paraformaldehyde plus 4% sucrose in PBS, pH 7.2, for 15 min at room temperature. Cells were then washed three times with cold PBS and permeabilized with 0.1% Triton X-100. The procedure was then followed as stated for tissue sections.

Glutamate Exposure

Induction of excitotoxicity in neurons was performed after 7 days in vitro (7DIV) by adding to the culture medium 300 μM glutamate or control buffer. After 5 or 10 min, the medium was completely replaced by fresh medium containing B27, and the neurons were incubated for an additional 20 hr before lysing them in NP-40 buffer.

Immunoblotting

Neurons were homogenized in NP-40 lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol), with protease inhibitors cocktail (Sigma), PMSF 1 mM, and sodium vanadate 10 mM. Cortical areas of animals subjected to CD or sham animals were homogenized in modified RIPA lysis buffer for tissues (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% SDS, and protease inhibitors). Proteins were determined using BSA as standard using the BCA assay (Pierce). Samples with equivalent protein content were separated by SDS-polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membrane. The membrane was blocked in Tris-buffered saline/Tween (10 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween 20) with 5% (w/v) dried skim milk powder added. The primary and secondary antibody incubations were carried out in the blocking solution. Immunoreactive bands were detected by using the enhanced chemiluminescence solution (Pierce) and AGFA films.

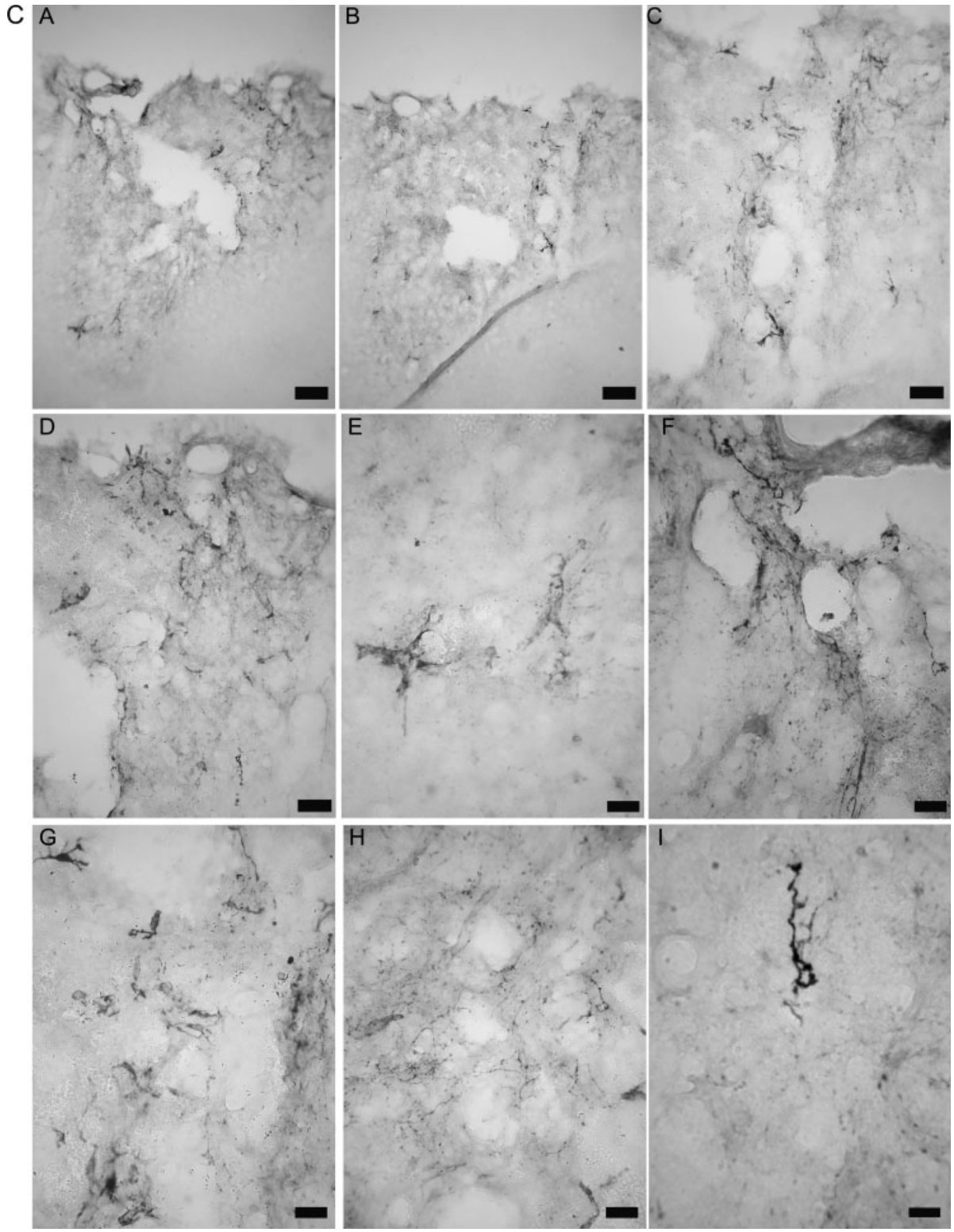
RESULTS

Expression of p75^{NTR} Is Induced in Isolated Cells After the Focal Ischemia

The CD paradigm used in this study induces a focal ischemic lesion in the brain cortex (Ramos et al., 2004). CD lesion resulted in physical disruption of the vasculature overlying part of the prefrontal cortex that produced a necrotic infarct (core) restricted to the lesion site, and a penumbra area was observed in the surrounding tissue (Fig. 1A). The depth of the lesions varied somewhat among animals, but typically this affected only cortical layers I–IV, giving a well-defined ischemic lesion with core and penumbra areas (Fig. 1B). The p75^{NTR} immunostaining was detected in isolated cells of the penumbra area after 1DPL and 3DPL (Fig. 1B). The morphology of p75^{NTR}-immunoreactive cells was characteristic of degenerating neurons showing an irregularly shaped soma and a reduced number of projections (Fig. 1C,D). Both polyclonal and monoclonal antibodies directed against the extracellular or intracellular p75^{NTR} domains were used, and the results obtained were identical (data not shown). The p75^{NTR}-positive cells were isolated and surrounded by negative cells as observed with nuclear Hoechst staining (Fig. 1B,D).

p75^{NTR} Expression Is Present at 1DPL and 3DPL but Is Reduced at 7DPL

The temporal profile of p75^{NTR} expression was analyzed by immunocytochemistry and immunoblot. The expression of p75^{NTR} was readily detectable at 1DPL, increased at 3DPL, and decreased by 7DPL (Fig. 2A,B). The p75^{NTR} immunostaining was observed in the ischemic penumbra in the hemisphere ipsilateral to the lesion and was absent in the contralateral cortex. The Sp1 transcription factor was expressed in brain hemispheres ipsilateral and contralateral to the ischemic lesion, but was



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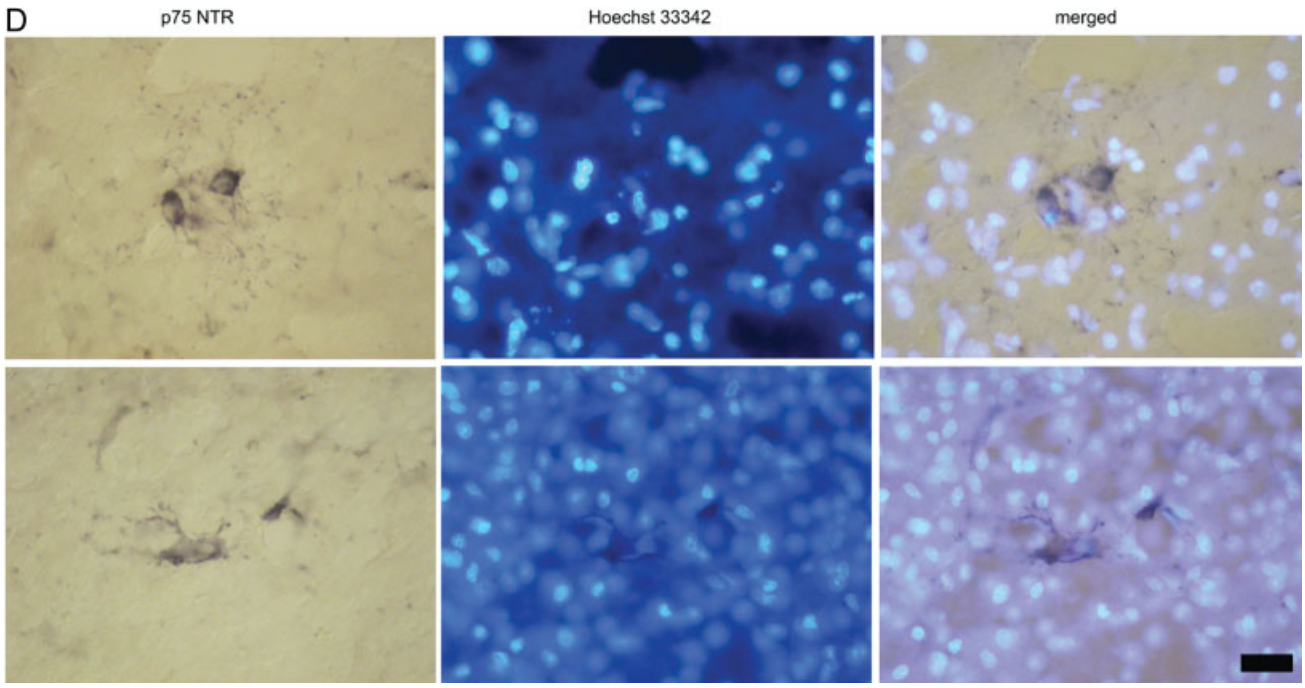

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clearly increased within the ipsilateral hemisphere at 3DPL and 7DPL (Fig. 2C).

The p75^{NTR+} cells showed nuclear localization of Sp1 transcription factor, but other p75^{NTR}-negative cells also showed Sp1 staining (Fig. 2D). Whereas p75^{NTR}/Sp1-positive cells were present both in the ischemic core and in the penumbra by 1DPL, p75^{NTR}/Sp1-positive cells were present only in the penumbra at 3DPL (Fig. 2D).

Neurons, but Not Astrocytes, Express p75^{NTR} and Sortilin After Ischemia by CD

The p75^{NTR} cells showed typical neuronal morphologies. To confirm the identity of p75^{NTR}-expressing cells, double immunostaining was performed with the neuronal marker NeuN. Figure 3A shows that both p75^{NTR} and NeuN are coexpressed in the same cell. The NeuN staining also showed numerous p75^{NTR}-negative neuronal bodies in the area of p75^{NTR} staining and very few p75^{NTR+} cells negative for NeuN (Fig. 3A) and also negative for glial fibrillary acidic protein (GFAP; data not shown). Hoechst nuclear staining, in agreement with these results, demonstrated a large number of p75^{NTR}-negative cells in the vicinity of p75^{NTR}-positive neurons (Fig. 2D). The expression of the p75^{NTR} coreceptor sortilin was also detected in p75^{NTR}-positive neurons (Fig. 3B).

p75^{NTR} Positive Neurons Are Also Positive for Markers of Apoptotic Cell Death

The p75^{NTR}-positive neurons have an abnormal morphology showing classical features of neuronal degeneration. p75^{NTR}-immunoreactive neurons presented large vacuoles in the cytoplasm and an irregular membrane perimeter (Fig. 1C). The nuclear morphology of p75^{NTR}-positive neurons showed condensed chromatin or images typical of apoptotic nuclear bodies (Fig. 2D). To confirm the apoptotic status of p75^{NTR}-positive neurons, double immunostaining for active caspase-3 and p75^{NTR} was performed. Figure 4 shows that p75^{NTR} is expressed by active caspase-3-positive neurons.

The Excitotoxicity of Glutamate Induces Massive p75^{NTR} Expression and Increased Sp1 Transcription Factor Levels in Primary Cortical Neurons

To shed light on the mechanism of induction of p75^{NTR} expression in neurons of ischemic penumbra, we exposed primary cortical neurons to an excitotoxic stimulus similar to what penumbra neurons received. Excitotoxicity in vitro was induced by glutamate exposure for 5 or 10 min, followed by extensive washing. A 5-min pulse of 300 μM glutamate induced robust p75^{NTR} expression in cultured cortical neurons (Fig. 5A). Because induction of neuronal p75^{NTR} expression by hyposmolar exposure depends on the reduced Sp1 degradation that is observed as increased Sp1 levels (Ramos et al., 2007), we analyzed the Sp1 abundance in primary neurons exposed to glutamate excitotoxicity. Excitotoxic stress significantly increased Sp1 content in neurons in a time-dependent manner (Fig. 5A). Immunocytochemistry of these excitotoxicity-exposed neurons

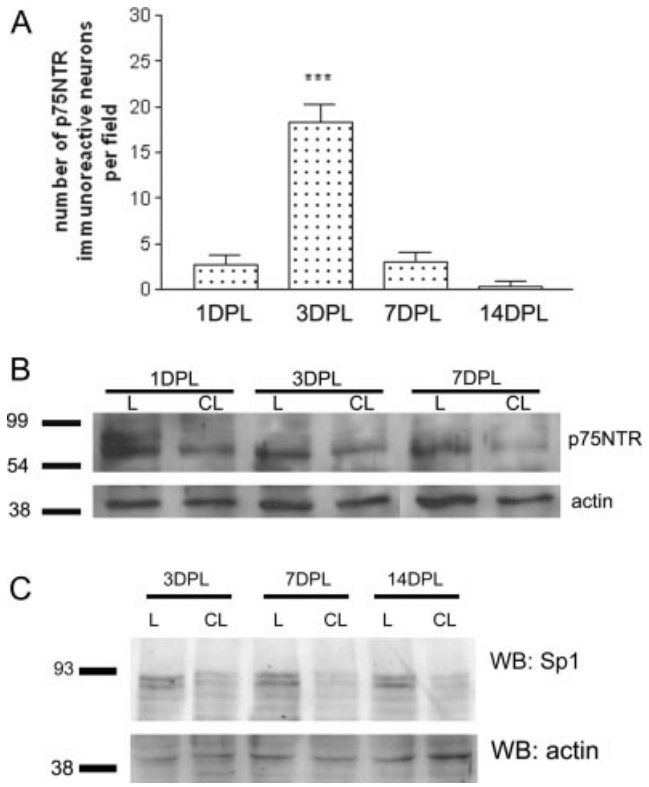

Fig. 2. **A:** Quantification of $p75^{NTR}$ cells in the cortex of the lesioned hemisphere (ipsilateral) at different time points after ischemia induced by cortical devascularization. The number of neurons per ×10 field was counted in ImageJ software (NIH) in five to eight sections per animal, four animals per group in each experiment. Representative data from one of four experiments are presented as mean number of neurons ± SD per field. In the brain cortex of the contralateral hemisphere, $p75^{NTR}$-immunopositive cells were not detected. **B:** Immunoblot showing the $p75^{NTR}$ expression in the lesioned and contralateral hemispheres at different time points after ischemia (days postlesion; DPL). Actin is shown as a loading control. **C:** Immunoblot showing the abundance of transcription factor Sp1 in the lesioned and contralateral hemispheres at different time points after ischemia by cortical devascularization. Actin is shown as a loading control. **D:** Double immunostaining showing the presence of Sp1 and $p75^{NTR}$ in the same cells. Note that expression of $p75^{NTR}$ is present at 1DPL and 3DPL in the ischemic penumbra (arrowheads). Nuclei of $p75^{NTR}$/Sp1-positive cells show chromatin condensation. Sp1 is a ubiquitous transcription factor, so it is also present in several $p75^{NTR}$-negative cells. ***$P < 0.001$. Scale bars = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

showed that $p75^{NTR}$ was expressed in most cells of the culture (Fig. 5B).

DISCUSSION

The dual role of $p75^{NTR}$ as inducer of neuronal survival and neuronal death has been established by a strong body of evidence (Barker, 2004; Nykjaer et al., 2005; Kalb, 2005). $p75^{NTR}$ is abundantly expressed during CNS development but is down-regulated in the adult. P75NTR is reexpressed in conditions of increased neuronal death, including mechanical damage, focal ischemia, axotomy, stroke, and Alzheimer's disease (Dechant and Barde, 2002).

In this work we found that a localized ischemic lesion to the brain cortex produced by the CD paradigm induced $p75^{NTR}$ expression in isolated neurons of the ischemic penumbra surrounding the ischemic core. After brain ischemia, neurons from the ischemic core die mainly by necrosis, with a secondary late neuronal death induced mainly by excitotoxicity that occurs in the ischemic penumbra. The proposed molecular events that occur in neurons exposed to ischemia are, first, a dramatic drop in oxygen and glucose supply, which turns neuronal metabolism into anaerobic conditions that are less efficient at producing ATP, and, finally, produces the depletion of ATP levels and blocks ATPases. The loss of membrane potential and the reversion of gradients determine the rapid cell swelling and cell death. These neurons usually belong to the core region, and their death produces a secondary massive release of excitatory amino acids and tissue inflammation and induces the astroglial reaction. These events induce secondary cell death (in the penumbra region) mediated mainly by excitotoxicity, which thus is reduced by ionotropic glutamatergic antagonists (Stone and Addae, 2002; Reinés et al., 2005; Molinuevo et al., 2005; Lipton, 2007). Our results show that $p75^{NTR}$-positive neurons after ischemia are apoptotic neurons, insofar as they showed NeuN staining and morphological characteristics of degeneration, including absence of nucleolus, shorter projections, condensed chromatin, and active caspase-3 staining. Surprisingly, not all dying neurons from the penumbra expressed $p75^{NTR}$. $p75^{NTR}$ specifically labeled some neurons. There are also a few NeuN-negative cells, with a clear neuronal morphology, that also express $p75^{NTR}$. It remains to be elucidated whether these cells are degenerated neurons that lost the NeuN staining, as was described previously (Robertson et al., 2006; Aviles et al., 2008), or another cell type in which $p75^{NTR}$ expression is also induced by ischemic lesion. $p75^{NTR}$-positive dying neurons were labeled with polyclonal and monoclonal antibodies directed against the extracellular and the intracellular domains, showing that $p75^{NTR}$ is likely to be active and properly expressed in these neurons. The maximal numbers of $p75^{NTR}$-positive, dying neurons were found at 1DPL and 3DPL, thus showing the potential $p75^{NTR}$ participation in delayed neuronal death that is induced in the penumbra. The specific localization of the $p75^{NTR}$ dying neurons in the penumbra area could also indicate that $p75^{NTR}$ expression is restricted to specific neurons that were damaged by the injury or that should be eliminated to preserve the function of the neuronal network. Why these neurons express $p75^{NTR}$ while their neighboring ones do not is still unknown.

The potential ligands for triggering $p75^{NTR}$-mediated neuronal death are the proneurotrophins, particularly pro-NGF (Harrington et al., 2004; Barker, 2004).

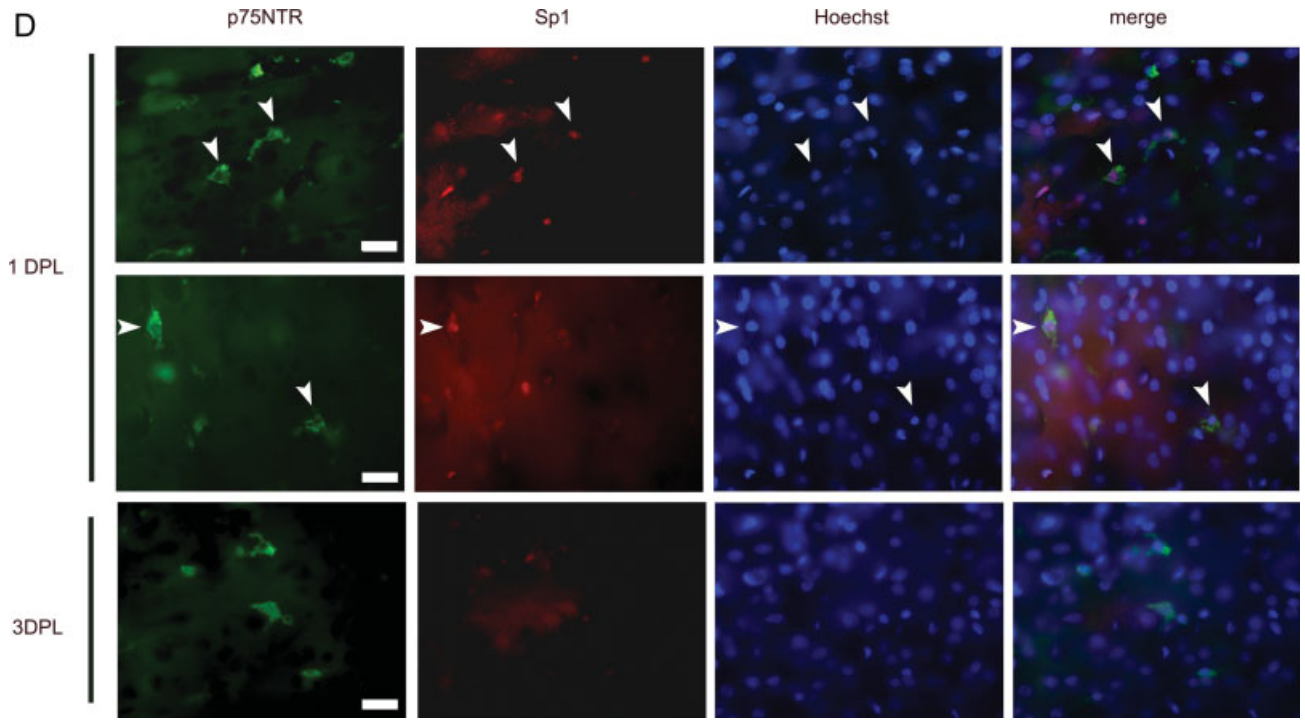

Figure 2. (Continued)

The disruption of the pro-NGF-p75^{NTR} signaling using knockout mouse models or blocking antibodies rescues corticospinal neurons in a p75^{NTR}-dependent manner (Harrington et al., 2004). Because pro-NGF is cleaved to the mature form intracellularly, the source of pro-NGF could be provided by cell rupture and/or deregulated secretion. In a model of photoreceptor loss after retinal injury, microglial cells are the main source of pro-NGF (Srinivasan et al., 2004). Pro-NGF extracted from injured spinal cord induced the apoptosis of cultured oligodendrocytes expressing p75^{NTR} (Beattie et al., 2002) and also seems to be produced by microglial cells (Yune et al., 2007). Reactive astrocytes secrete pro-NGF into the culture medium, and that medium induces neuronal death of motor neurons in a pro-NGF-dependent manner (Domeniconi et al., 2007). The extensive astroglial reaction induced by CD was demonstrated previously (Ramos et al., 2004) and is likely to be the main source of immature proneurotrophins inducing neuronal death of p75^{NTR}-positive neurons in our model of ischemia.

In addition, it is clear that intracellular adaptors required for the neuronal death induction are also present and even up-regulated after CNS injury (Ramos et al., 2007; Arnett et al., 2007). Sortilin mRNA and immunoreactivity both displayed a widespread distribution throughout the brain (Sarret et al., 2003). Sortilin expression was found in neuronal cell bodies and dendrites of the piriform cortex, hippocampus (Sarret et al., 2003), and retina after ischemia induced by ocular pressure (Wei et al., 2007). In fact, the p75^{NTR}-positive neurons that we detected in the ischemic penumbra are also positive for sortilin. In this scenario, the control of p75^{NTR} expression becomes in a main player in determining cell fate after CNS injury. The control of p75^{NTR} expression emerges as one of the late checkpoints in the induction of cell death. The participation of the Sp1 transcription factor in p75^{NTR} expression has been determined, and the mechanism proposed for the increased Sp1 abundance involves the blockage of proteosomal Sp1 degradation (Ramos et al., 2007). Decreased turnover of Sp1 could be a determinant inducing the p75^{NTR} expression (Ramos et al., 2007). In agreement with this hypothesis, we found that Sp1 abundance is increased in our model of ischemia induced by CD. In addition, Sp1 transcription factor is accumulated in the nucleus of p75^{NTR+} neurons in vivo and increases the abundance in excitotoxicity-exposed primary cortical neurons in vitro. Because p75^{NTR} can be dynamically regulated, a plausible function of the p75^{NTR}–proneurotrophins interaction is to eliminate damaged cells that express p75^{NTR} (Pehar et al., 2005).

A remarkable characteristic of ischemic penumbra is the secondary excitotoxic cell death induced by increased level of glutamate. In fact, our in vitro studies in primary cortical neuronal cultures showed that excitotoxic stress was able to induce p75^{NTR} expression. The increased expression of p75^{NTR} was present in most cells

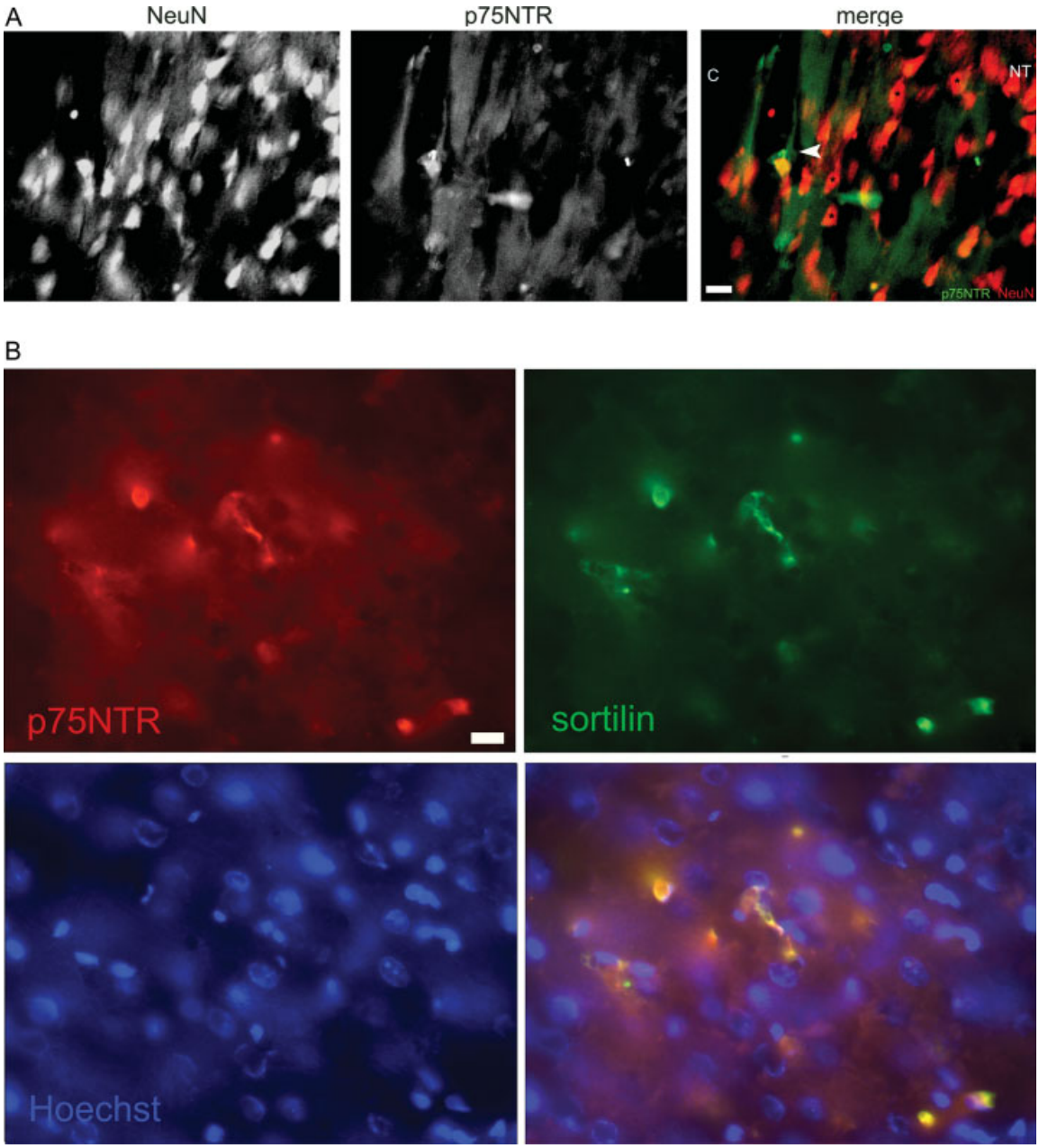

Fig. 3. **A:** Double immunostaining showing NeuN (red) and $p75^{NTR}$ immunoreactivity (green) at 3 days postlesion (3DPL). The photograph shows the ischemic core (C), the normal tissue (NT), and the penumbra area where many NeuN-positive neurons are present (asterisks) as well as $p75^{NTR}$-positive neuron costained with NeuN (arrowhead). **B:** $p75^{NTR}$-positive neurons are also positive for sortilin. The photograph shows $p75^{NTR}$-positive neurons colabeled with the receptor sortilin. Scale bars = 7 μm in A; 20 μm in B. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of the culture, a noticeable difference from in vivo studies showing isolated neurons labeled with $p75^{NTR}$. Increased Sp1 abundance has also been observed in primary neurons exposed to excitotoxity, reflecting a mechanism similar to that reported for hyposmolar stress (Ramos et al., 2007).

The results presented here may support the hypothesis that $p75^{NTR}$ expression destines neurons to die after brain ischemia. This result has also been demonstrated in epileptic seizures in which apoptotic neurons were labeled with $p75^{NTR}$ (Roux et al., 1999). In our model of ischemia, the specific localiza-

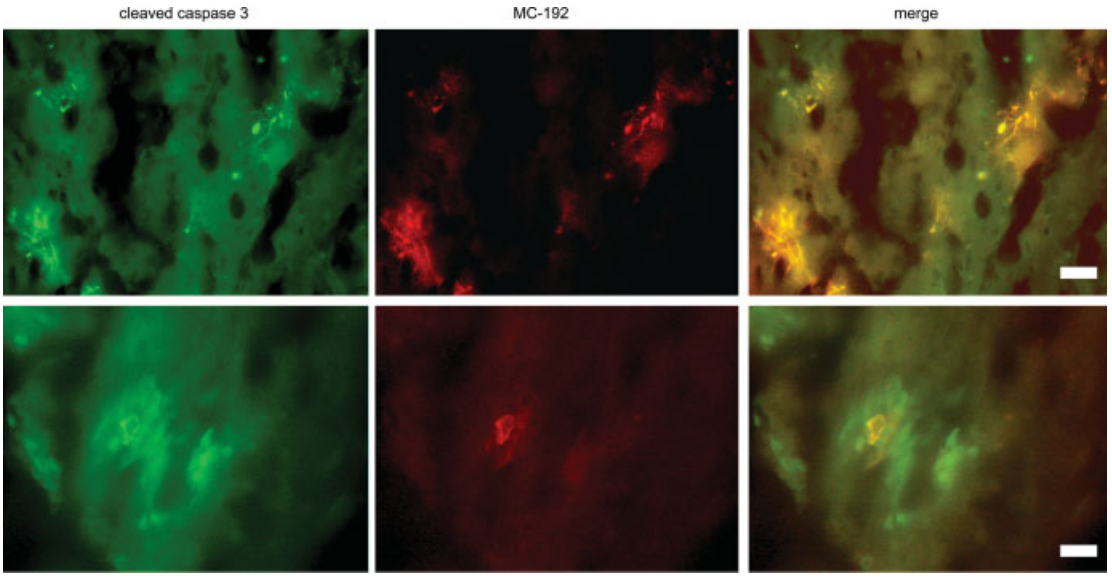

Fig. 4. Double immunostaining showing coexistence of cleaved caspase-3 (green) and $p75^{NTR}$ (red). The anti-$p75^{NTR}$ antibody (clone MC192) is directed against the extracellular domain (ECD) of $p75^{NTR}$ and labels soma but also a number of projections and dendritic arborization in detail. Scale bar = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

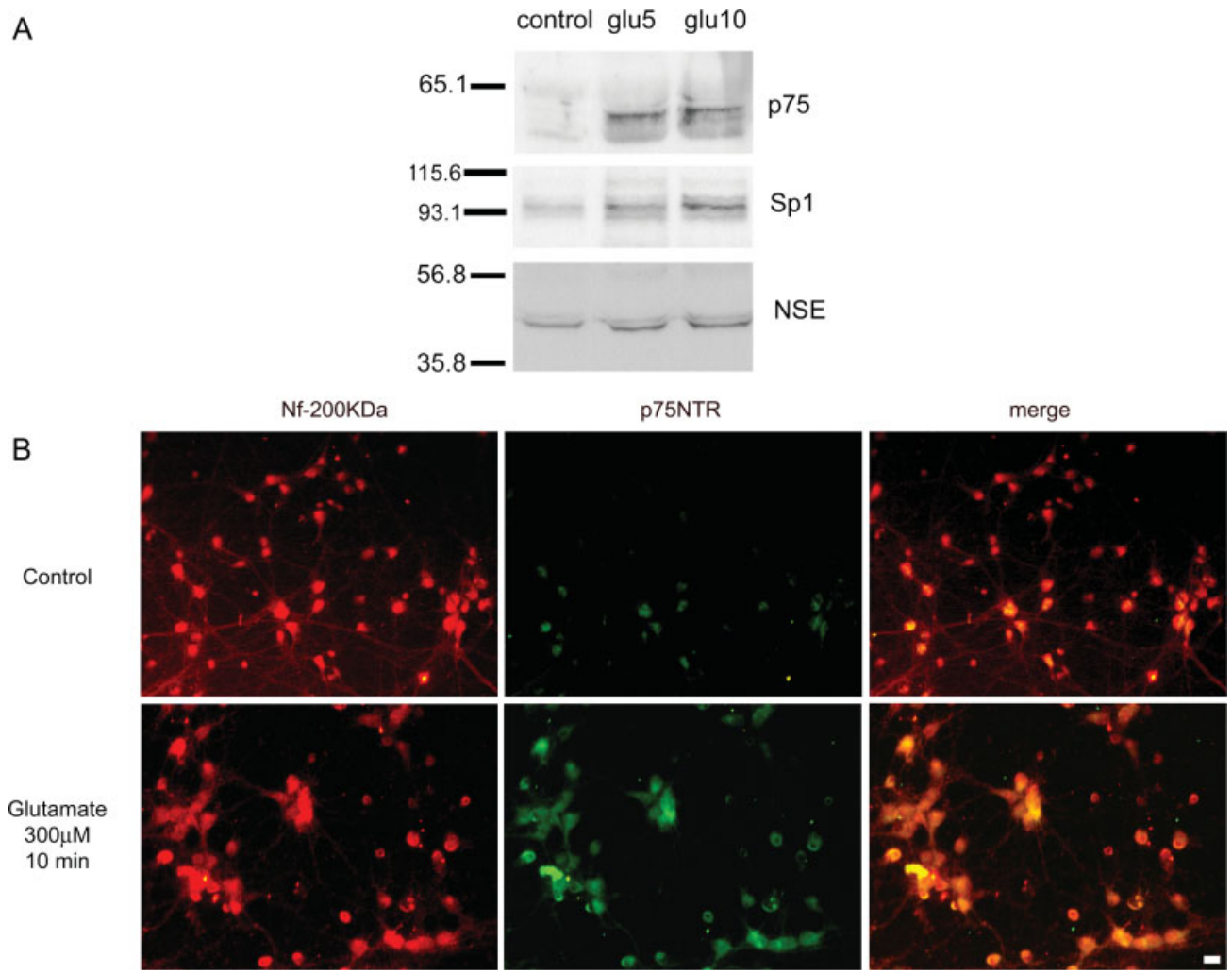

Fig. 5. **A:** Immunoblot analysis of primary cortical neurons (7DIV) exposed for 5 or 10 min to an excitotoxic stimulus of 300 μM glutamate (glu5 or glu10, respectively) and lysed 24 hr later. NSE, neuron-specific enolase, used as loading control. **B:** Double immunostaining of $p75^{NTR}$ and neurofilament (200 kDa), specific neuronal marker in 7DIV cortical neurons exposed to the excitotoxic stimulus of 10-min glutamate pulse and stained 24 hr later. Note the features of neuronal degeneration in glutamate-exposed cells, including shorter projections, irregular cell limits, and absence of a nucleolus. Scale bar = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tion of $p75^{NTR}$ neurons in the penumbra reflects that $p75^{NTR}$ expression may be a determinant in the secondary wave of neuronal death that follows the necrotic death of the ischemic core. In accordance with our in vitro results, glutamate excitotoxicity is a main candidate for inducing $p75^{NTR}$ expression in the penumbra, although hyposmolar stress and cell swelling are also powerful inducers of $p75^{NTR}$ expression (Ramos et al., 2007). In vivo availability of proneurotrophins and binding partners such as sortilin makes the $p75^{NTR}$ expression one of the last steps in the events leading to neuronal death.

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