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Study of the Role of the Low-Affinity Neurotrophin Receptor p75 in Naturally Occurring Cell Death during Development of the Rat Retina

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Key Words
p75 · Rat · Retina · Apoptosis · Neurotrophin · Development

Abstract
The aim of this study was to examine the tempo-spatial expression of low-affinity neurotrophin receptor p75, or $p75^{NTR}$, and its role in the induction of retinal ganglion cell (RGC) apoptosis in the rat retina during development. The cellular distribution of p75 in the retina was demonstrated with immunohistochemistry and double-immunofluorescent staining. Apoptosis in the developing rat retina was detected by DNA gel electrophoresis, and the number of RGCs undergoing apoptosis was estimated by terminal deoxyribonucleotidyl-mediated dUTP-digoxigenin nick end labeling (TUNEL). To localize p75 on apoptotic RGCs, p75 immunofluorescence and TUNEL fluorescent staining was performed on sections with Fluoro-Gold-prelabeled RGCs. p75 immunoreactivities were not detected either on the RGCs or TUNEL-positive cells, whereas Müller cell processes were p75 immunopositive. Thus, it was most unlikely that p75 induced apoptosis of RGCs in the rat retina.

Introduction

It is well known that neurotrophins, such as nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin-3 (NT-3) and neurotrophin-4/5, can support the survival and differentiation of neurons during development and enhance regeneration after injury. Neurotrophins bind to two kinds of receptors, high-affinity tyrosine kinase receptors (TrkA, TkrB and TrkC) and the low-affinity neurotrophin receptor p75. The low-affinity neurotrophin receptor p75 is a 75-kD cell surface glycoprotein that binds all neurotrophins with similar affinity but with different kinetics and is thought to help to ensure the specificity of each neurotrophin.

During retinal development in rodents, the ganglion cell layer (GCL) and optic nerve transiently express high levels of p75 receptor [Yan and Johnson, 1988; Carmignoto et al., 1991; Takahashi et al., 1993; Allendoerfer et al., 1994; Frade and Barde, 1999]. p75 immunoreactivity is found in the internal layers and GCL of the retina from embryonic day 16 (E16) to postnatal day 10 (PN10) in the rat [Yan and Johnson, 1988]. By using Northern blot analysis, p75 mRNA has been detected in the rat retina during the embryonic and postnatal periods [Takahashi et al., 1993]. The mRNA of p75 is found in the GCL, inner

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nuclear layer and inner plexiform layer during development by in situ hybridization [Koide et al., 1995; Ugolini et al., 1995]. In rat retina, p75 has been reported to be presumably localized on both the retinal ganglion cells (RGCs) [Carmignoto et al., 1991; Hammes et al., 1995] and Müller cells [Schatteman et al., 1988; Chakrabarti et al., 1990] at the light microscopic level. We have demonstrated that p75 is localized on the Müller glia processes and not on RGCs in normal, injured and regenerating adult rat retinae by means of immunocytochemistry at both light and electronic microscopic levels [Hu et al., 1998, 1999].

Naturally occurring cell death of neurons and glia is a normal process during the development of the vertebrate retina. Cell loss in the retina under both normal and abnormal developmental conditions is correlated with apoptosis [Linden et al., 1999]. Among the apoptotic and antiapoptotic inducing genes involved in retinal cell death, a constitutive promoting role of p75 in cell death has been proposed [Rabizadeh et al., 1993; Rabizadeh and Bredesen, 1994]. p75 contains a protein with a death-associated domain that is also found in the cytoplasmic region of tumor necrosis factor and Fas receptors [Feinstein et al., 1995]. A possible involvement of p75 in programmed cell death during early development has been suggested [Cortazzo et al., 1996; Van der Zee et al., 1996]. In the early development of the chick [Frade et al., 1996] and mouse [Frade and Barde, 1999] retina, p75 has been demonstrated to show a proapoptosis function in retinal neurons. In contrast, our observation on the topographic expression of p75 in normal adult injured and regenerating rat retinae indicates that p75 may play in indirect role in RGC protection [Hu et al., 1998, 1999].

Even though RGCs undergo naturally occurring cell death during development and there is no evidence that apoptotic RGCs express p75 in the adult rat retina, it is of interest to understand whether RGCs undergoing apoptosis during development express p75. The present study was undertaken to examine the role of p75 in mediating apoptosis of RGCs during the development of the retina.

Materials and Methods

Experimental Animals

The experiments were performed on embryonic, postnatal and young adult female (180–250 g) Sprague-Dawley rats. Young female Sprague-Dawley rats with positive vaginal plug (or positive sperm smear) were recorded as first day of gestation or E1. Newborn animals less than 24 h old were taken as PN1.

Northern Blot Analysis

Total retinal RNA was extracted from E14 (n = 24), E18 (n = 24), PN1 (n = 30), PN7 (n = 20), PN14 (n = 18), PN21 (n = 20) and 8-week-old adult (n = 20) rats by Trizol reagent following the manufacturer's instructions. The 1.151-kb *Pvu* II fragment of p75 cDNA and a 1.4-kb fragment of β-actin were used as templates. About 2–4 μg of mRNA of each developmental stage were loaded onto 1.2% agarose/formaldehyde gel. After electrophoresis, the samples were transferred to Hybond-N^+ Nylon membrane sheets (Amersham). The membrane was first prehybridized at 65°C in ExpressHyb hybridization buffer (Clontech) for 30 min. Denatured [α^{32}P]-dCTP-labeled probe was then added to the replaced fresh hybridization buffer. After incubation for 1 h, the membrane was washed three times with 2 × sodium chloride/sodium citrate (SSC) (buffer) plus 0.05% SDS for 40 min at room temperature. The membrane was further washed with 0.1 × SSC plus 0.1% SDS twice for another 40 min at 50°C. After washing, the membrane was wrapped with a plastic sheet and exposed in a cassette (Molecular Dynamics) for 1–2 days. Images were read by a Phosphor Imager scanner and the results of Northern blot were analyzed by ImageQuant (Molecular Dynamics). The relative mRNA levels of p75 were normalized to the β-actin mRNA level. The membrane was also exposed to X-ray film for a permanent record.

Retrograde Labeling of RGCs with Fluoro-Gold

Animals were operated on under chloral hydrate anesthesia (7% aqueous solution, 0.6 ml/100 g body weight, intraperitonally). Neonatal animals (PN1–PN7) were anesthetized by hypothermia. The superior colliculus (SC) was exposed after removing the connective tissues or membranes over it. Fluoro-Gold (FG; 6%) dissolved in saline was applied to the SC using a glass micropipette. A piece of gelfoam socked with FG was then left on the surface of the SC before the bone flap was folded back. The skin was sutured with 5/0 surgical silk. Quinine sulfate dissolved in PBS was applied at the site of the sutured skin to prevent the mother from eating the pups. Usually, a 3-day period is given for the maximum FG retrograde labeling of RGCs. The efficiency of FG in labeling RGCs has been described previously [Ng et al., 1995; Hu et al., 1998] in our laboratory.

Tissue Preparation

The animals, whether the retinae were prelabeled with FG or not, were deeply anesthetized with chloral hydrate, perfused with normal saline followed with 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4). The eyeballs were postfixed in the same fixative overnight at 4°C. For embryonic tissues, pregnant rats were killed by decapitation, and embryos were quickly removed from the uterus. All the embryos were fixed overnight by immersion in fixative as described above. The nonprelabeled eyeballs were dehydrated in graded alcohol, cleared and embedded in paraffin. Parasagittal paraffin (4-μm) sections were collected onto gelatin-coated slides, air dried and processed for histology. Prelabeled eyeballs were transferred into 10% and then 30% sucrose in 0.1 *M* phosphate buffer overnight at 4°C. Cryosections (10–15 μm) were prepared for immunofluorescent or terminal deoxyribonucleotidyl-mediated dUTP-digoxigenin nick end labeling (TUNEL) staining. Some retinae were dissected out as whole mounts for p75 immunohistochemistry.

Immunohistochemistry

p75 immunohistochemistry on retinal paraffin or frozen sections and whole mounts has been described in detail previously [Hu et al.,

1998, 1999]. The standard avidin-biotin-complex-3,3′-diaminobenzidene (ABC-DAB) method was performed on parasagittal paraffin sections. Briefly, the sections were dewaxed, rehydrated and then incubated in 0.1% hydrogen peroxide for 30 min. This was followed by incubating the sections in 10% normal horse serum (HS), 10% rat serum (RS) and 5% BSA in PBS for 1 h, and subsequently with 192-IgG (1:50; Oncogene) or mouse anti-vimentin IgG (1:200; Sigma) overnight at 4°C. Biotinylated horse anti-mouse IgG (1:250; Vector) was applied to the sections for 1 h at room temperature, followed by ABC complex (Vector) or avidin-FITC (1:1,000; Vector) for 45 min. The color was then developed with freshly prepared DAB-cobalt chloride solution (0.03% DAB, 0.017% cobalt chloride) containing 0.01% hydrogen peroxide. The sections were rinsed in PBS between each step. Double staining enhancer (DS kit from Zymed Laboratories Inc.) was used in the double indirect immunofluorescent staining. Finally, the sections were counterstained with neutral red, dehydrated, cleared, mounted in Permount for paraffin sections and examined under a light microscope or mounted in glycerol and observed under the fluorescent microscope with appropriate filters (MM-11 Nikon, Japan).

The indirect p75 immunofluorescent procedure was applied to FG-prelabeled retinal whole mounts. Briefly, after blocking nonspecific staining using 20% heat-inactivated HS, 10% RS, 5% BSA and 2% Triton X-100 in 0.01 *M* PBS for 1 h, the retinae were incubated for 3 nights at 4°C in PBS containing mouse anti-rat 192-IgG monoclonal antibody, 5% HS and 2% Triton X-100. The retinae were then incubated overnight in washing buffer (PBS containing 1% HS, 1% RS and 0.3% Triton X-100) containing biotinylated horse anti-mouse IgG (1:250, rat absorbed; Vector) and transferred to PBS containing FITC-avidin conjugate (1:1,000; Vector) for 2 h. The retinae were rinsed in washing buffer between each step. After mounting the retinae in glycerol, FITC-positive staining as well as FG-labeled RGCs were observed under a Nikon or Olympus fluorescence microscope with appropriate filters.

DNA Extraction and Gel Electrophoresis

Retinal DNA was extracted according to a method described previously which eliminates high-molecular-weight DNA and increases the efficiency of the electrophoretic detection of small DNA fragments [Hockenbery et al., 1990; Cook et al., 1998]. Briefly, retinae (n = 16) from each time point (PN1, PN3 and PN5) were isolated and homogenized in lysis buffer (0.5% Triton X-100, 5 m*M* Tris buffer, pH 7.4, 20 m*M* EDTA) at 4°C for 20 min. After centrifugation at 27,000 *g* for 15 min, supernatants were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated in ethanol and centrifuged. The pellet was resuspended in 20 μl of tris/EDTA (TE) buffer and subsequently treated with RNAase for 1 h at room temperature. DNA samples were electrophoresed on a 1.5% agarose gel containing ethidium bromide (0.4 μg/ml).

TUNEL Staining

The ApopTag peroxidase kit (Oncor, S7100) was applied to detect cells undergoing programmed cell death. The staining procedure was slightly modified from the manufacturer's suggestion on the retinal paraffin sections. For FG-prelabeled retinal cryostat sections, anti-digoxigenin-rhodamine (5 μg/ml, Boehringer Mannheim) was used to substitute the anti-digoxigenin-peroxidase in the S7100 kit in order to generate red fluorescence.

Double-Fluorescent Staining with TUNEL and p75 Immunohistochemistry

TUNEL fluorescent staining (described above) on neonatal rat retinal frozen sections was performed first, followed by p75 indirect immunofluorescent staining (the same as described above). The sections were mounted in glycerol and examined under a Leica laser confocal microscope.

Data Analysis

Parasagittal sections with TUNEL staining were selected for quantitative analysis. Only the sections cut close to the optic disc or with the optic nerve head were used for cell counting. Three retinae were used for each time point. For each retina, 4–6 sections were selected randomly for counting. All TUNEL-positive cells located in the GCL or outer nuclear layer were counted for each section under × 40 magnification with an eyepiece graticule. This sampling method was modified from a previous study [Tso et al., 1994].

The number of counted cells from all sections was averaged for each retina. Then, the means from the numbers of cells from three retinae were taken to represent the corresponding time point. The SPSS/PC$^+$ statistical package was used for data analysis. One-way variable analysis (one-way ANOVA, Duncan's test) was performed for all the counting data.

Results

Gradual Increase in the Expression of p75 mRNA in the Retina during Development

The expression of p75 during development was estimated at the transcription level by Northern blot analysis. The p75 mRNA expression levels were normalized to β-actin levels. p75 mRNA was expressed at a very low level during the early development of the retina from E14 to PN7 (from 32.7 to 113.6%). Although the level of p75 mRNA gradually increased during the developmental period, there was a much larger increase after PN7. The p75 mRNA level was almost two times higher at PN14 (233.3%) than at PN7. The expression of p75 mRNA reached its highest level in adult rats (475.0%) (fig. 1).

Expression of p75 in Embryonic and Neonatal Rat Retinae

In the parasagittal paraffin sections, p75 immunoreactivity was absent at E16 (fig. 2A), then first appeared in the GCL and neuroblast layer (NBL) at E18 (fig. 2B). The density increased in the GCL at PN1 (fig. 2C). This finding is consistent with previous reports [Yan and Johnson, 1988]. However, very little p75 immunoreactivity was seen in the internal layers. Although immunoreactivity of p75 in the GCL was high at PN1, the staining seemed to be restricted to the Müller cell processes that surrounded the somas of RGCs and was not found on the RGC somas themselves.

Retinal whole mounts from PN1, -3, -7, -14, -21 and -28 animals were processed for indirect 192-IgG immunofluorescent staining. No p75-positive staining was found on the FG-prelabeled RGCs in the GCL at any of the time points examined. p75 immunoreactivity was localized on the Müller cell processes around the RGCs (fig. 3). On frozen parasagittal retinal sections from different postnatal stages, p75 immunofluorescent staining (Texas Red, fig. 4B) was confined to Müller cell processes as shown by FITC (fig. 4C) around the FG-prelabeled RGCs (fig. 4A). This was in agreement with a previous finding that described p75 immunoreactivity on Müller cells but not on RGCs in primates [Schatteman et al., 1988]. The result is also consistent with our observations in both normal adult [Hu et al., 1998] and regenerating [Hu et al., 1999] rat retinae, but is in contrast to other in vivo studies. However, in these studies, the RGCs were not positively identified by retrograde prelabeling of RGCs [Carmignoto et al., 1991]. In addition, no significant change in p75 immunoreactivity was found among the postnatal animals during development.

Apoptosis in the Retina during the Period of Naturally Occurring Cell Death

An agarose gel electrophoresis of DNA extracted from PN1, PN3 and PN5 retinae showed that there was a ladder of DNA fragments at intervals of 180–190 bp. The result confirms that apoptosis occurred in developing rat retinae (fig. 5).

To localize the specific cell type undergoing apoptosis in the retina, a highly sensitive terminal transferase technique (TUNEL) for in situ detection of DNA strand breaks was employed in this study [Gavrieli et al., 1992; Frade and Barde, 1999]. The retinae of newborn rats have two major cellular strata. The innermost cellular stratum is the GCL. Immediately after birth (PN1), this layer contains only ganglion cells. The other stratum is the NBL [Linden et al., 1999]. FG-labeled RGCs that were TUNEL positive were found in the PN1 retina (fig. 6).

The Topographic Relationship between p75 and Apoptotic Cells

Double-fluorescent staining with TUNEL (rhodamine) and p75 immunohistochemistry (FITC) was performed on the same retinal parasagittal section. The absence of yellow fluorescence indicates that there was no overlapping of the TUNEL-positive (red) and p75-immunoreactive staining (green), as seen by means of a laser confocal microscope (fig. 7).

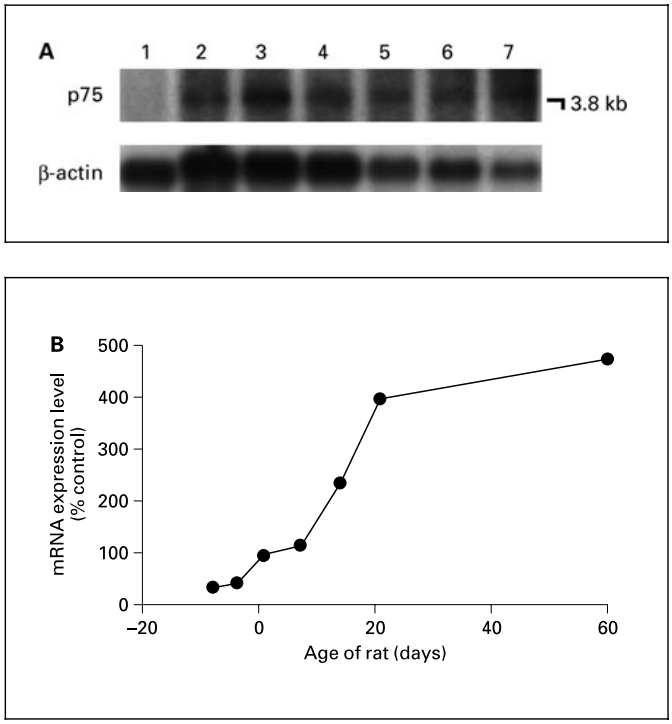
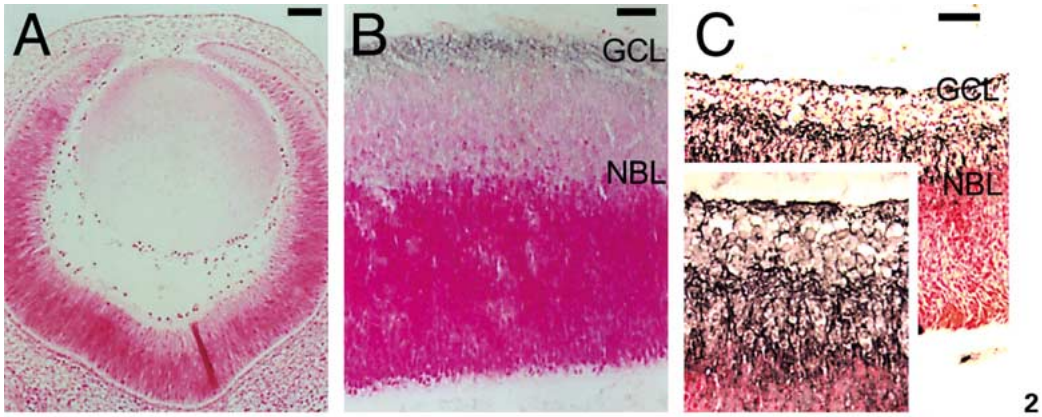
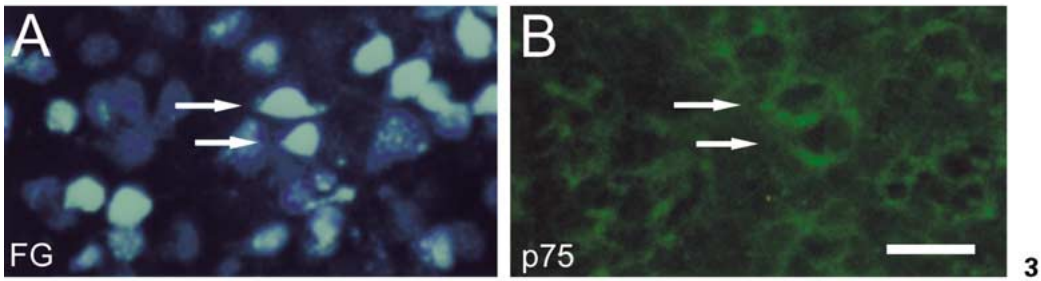


Fig. 1. Quantitative analysis of p75 mRNA levels in the developing retina by Northern blot hybridization. **A** Lanes 1–7 represent different developmental stages: 1 = E14; 2 = E18; 3 = PN1; 4 = PN7; 5 = PN14; 6 = PN21; 7 = adult. **B** Expression levels of p75 transcripts indicated as a percentage of their optical density normalized to the corresponding optical density of an internal control of β-actin mRNA. The p75 mRNA levels increased gradually during rat retina development.

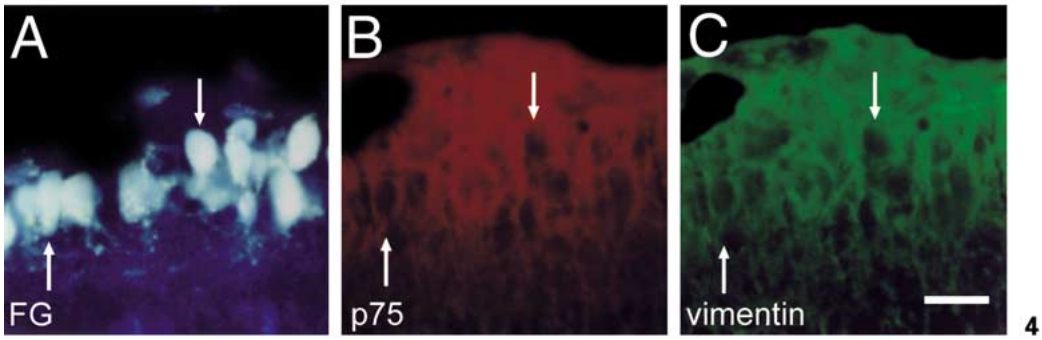
Relatively more TUNEL-positive cells (apoptotic cells) were found in the PN1 and PN3 retinae than in the embryonic and other postnatal retinae (fig. 5, 8). The apoptotic RGCs reached a peak at PN1 ($p < 0.05$, Duncan's test, one-way ANOVA; fig. 8). The number of apoptotic RGCs decreased after PN1, whereas the number of TUNEL-positive photoreceptor cells remained high at PN3 ($p < 0.01$, Duncan's test, one-way ANOVA). Most of the TUNEL-positive cells in the NBL at PN3 are photoreceptor cells. A few TUNEL-positive cells were found in the GCL (fig. 8). The DNA laddering gel (fig. 5) correlated very well with the total number of TUNEL-positive cells in the retinal sections (fig. 8). Indeed, a major component of apoptotic cells in the retina came from the photoreceptors, which peaked around PN3 (fig. 8). This is reflected by the remarkable DNA fragmentation shown by the laddering gel at PN3 (fig. 5).


A
B
GCL
NBL
C
GCL
NBL

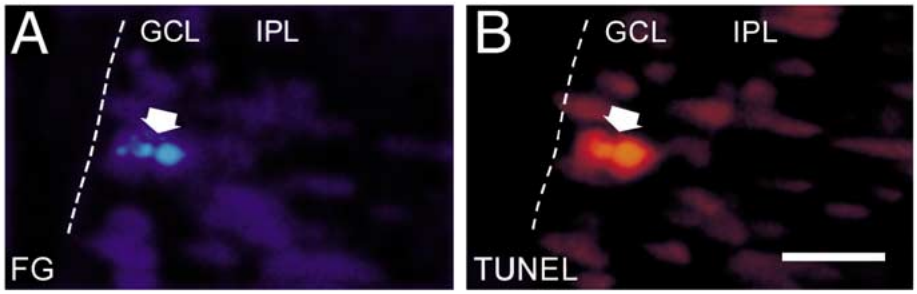
2


A
FG
B
p75

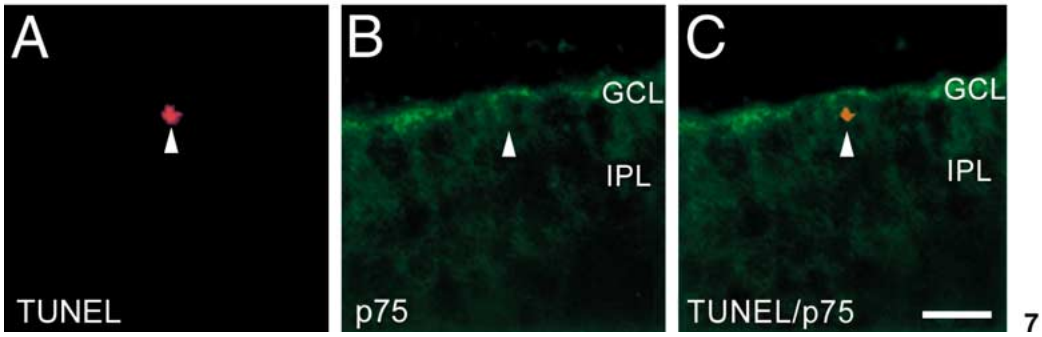
3


A
FG
B
p75
C
vimentin

4


A
GCL
IPL
FG
B
GCL
IPL
TUNEL

6

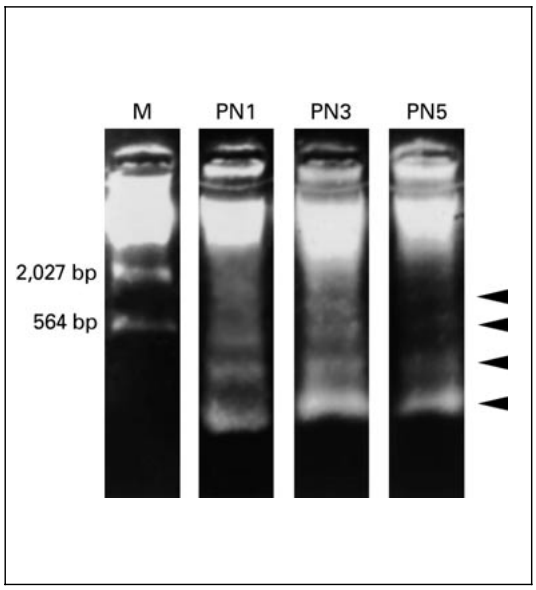

A
TUNEL
B
GCL
IPL
p75
C
GCL
IPL
TUNEL/p75

7

Discussion

Our studies focused on the tempo-spatial expression of p75 in the developing retina of Sprague-Dawley rats and its potential role in the induction of RGC apoptosis during development. The developing RGCs undergo apoptosis, which was confirmed by in situ TUNEL staining in this study. As p75 expression was not detected on apoptotic RGCs and was merely present around FG-prelabeled RGCs, as revealed by 192-IgG immunohistochemistry, it can be concluded that the expression of p75 is not directly correlated with the naturally occurring cell death of RGCs during development.

The low-affinity neurotrophin receptor p75 has generated a great deal of interest and controversy; however, the precise role of this receptor has remained obscure until recently [Bothwell, 1996; Barrett, 2000]. Accumulating evidence has suggested that p75 is a multifaceted receptor capable of fulfilling a wide number of biological functions [Rabizadeh et al., 1993; Barrett and Bartlett, 1994; von



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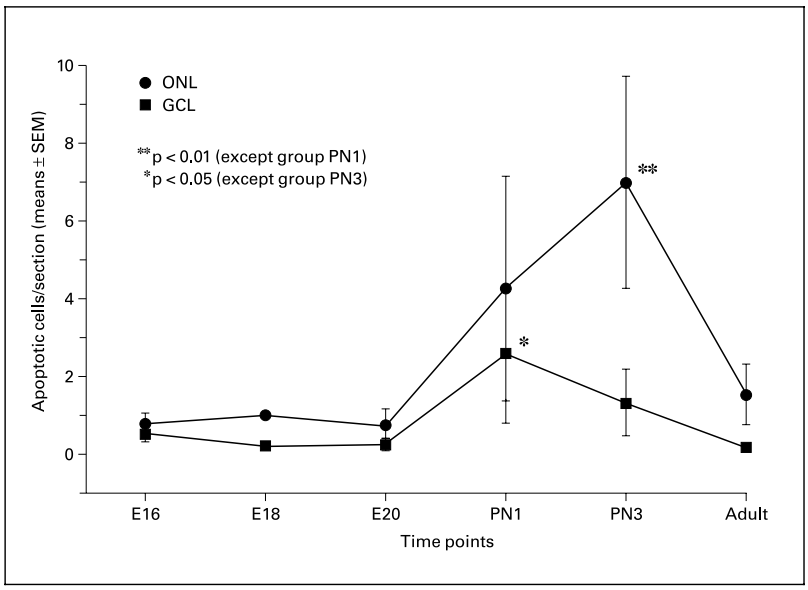

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Fig. 2. Color photographs showing p75 immunoreactivity in E16, E18 and PN1 paraffin sections (4 μm) counterstained with neutral red. p75 staining (dark-brown products) was absent at E16 (**A**), but appeared at E18 (**B**) and with increasing intensity at PN1 (**C**). The insert in **C** shows a high magnification of p75 staining in the GCL in the PN1 retina. The scale bar is 80 μm in **A** and 20 μm in **B** and **C**.

Fig. 3. 192-IgG immunoreactivity in a PN1 rat retinal whole mount with FG-prelabeled RGCs in the GCL. Scale bar = 20 μm. **A** FG-labeled RGCs were clearly shown in the GCL. **B** 192-IgG-immunopositive staining (FITC). Note that the FITC-immunopositive staining was absent in some blank holes (arrows in **B**) where FG-labeled RGCs (arrows in **A**) were located.

Fig. 4. Color photographs showing double-immunofluorescent staining on PN1 cryostat parasagittal sections of the retinal GCL. Scale bar = 20 μm. The same section was used to show the retrogradely labeled RGCs with FG (**A**), 192-IgG (Texas Red) (**B**) and vimentin (FITC) (**C**) immunopositive staining. Note that the Texas Red and FITC immunopositive staining were absent in some blank holes (arrows in **B** and **C**) where FG-labeled RGCs (arrows in **A**) were located. The arrows illustrate the location of the same pair of cells in all photographs.

Fig. 5. DNA gel electrophoresis from rat retinae of different developmental ages. The DNA ladders characterizing apoptosis are marked by arrowheads. M = DNA marker (λDNA/*Hin*d III).

Fig. 6. Double-immunofluorescent TUNEL staining on cryostat parasagittal sections of a PN1 retina. **A** FG-prelabeled RGCs. **B** Rhodamine-TUNEL-positive apoptotic RGCs. Scale bar = 20 μm. FG-prelabeled RGCs (arrow in **A**) showed DNA fragmentation, which was confirmed by rhodamine-TUNEL staining (arrow in **B**). IPL = Inner plexiform layer. The broken lines roughly represent the inner limiting membrane.

Fig. 7. Color photographs showing double-fluorescent staining with TUNEL and p75 immunohistochemistry in a PN1 retinal section. Scale bar = 20 μm. **A** A TUNEL-positive cell labeled with rhodamine (red) in the GCL indicated by an arrowhead. **B** p75-immunopositive staining (FITC) was found around a blank hole representing an RGC (arrowhead). **C** A double exposure of rhodamine (red) and FITC (green). Note that there is no overlapping between TUNEL- and p75-positive staining, since the color did not turn yellow (red and green), as indicated by the arrowhead. IPL = Inner plexiform layer.

Fig. 8. The number of apoptotic cells in the developing retina. Apoptotic RGCs showed a peak at PN1 during the period of naturally occurring cell death in the retina ($p < 0.05$, Duncan's test, one-way ANOVA). ONL = Outer nuclear layer.

Bartheld et al., 1994; Dobrowsky et al., 1994; Carter et al., 1996; Barrett, 2000]. So far, the data suggest that cells can transduce the NGF signal via p75, which could lead to apoptosis in a cell type-specific manner. On the other hand, data from the retina [Frade et al., 1996] suggest that cells in very early development, the precise identities of which are not clear (i.e. postmitotic neurons or undifferentiated neuronal precursors), are susceptible to NGF/p75-induced death. However, there is clearly no close correlation between the time of p75 expression by neurons and the time of the critical period of neuronal death during development. The data accumulated thus far would suggest that in order to make predictions about which cell type would die at a particular time, information is needed on: (1) the expression of p75; (2) the local concentration of binding neurotrophins as a function of time; (3) the level of expression of TrkA, -B and -C, along with the ratio of p75 to Trk expression, and (4) the expression of other antiapoptotic genes such as bcl-2, as well as additional determinants of cellular survival [Rabizadeh and Bredesen, 1994].

Although the signal transduction mechanisms of p75 in mediating cell death remain largely unknown, recent studies have shown that neurotrophin binding to p75 activates the ceramide pathway in a glia cell line [Dobrowsky et al., 1994; Bothwell, 1996; Dechant and Barde, 1997] and NF-κB in cultured Schwann cells [Carter et al., 1996]. It was clearly demonstrated that p75 may trigger cellular responses without the participation of Trk receptors at all, and that p75 signaling is of relevance in vivo [Carter et al., 1996; Casaccia-Bonnefil et al., 1996; Frade et al., 1996; Van der Zee et al., 1996; Dechant and Barde, 1997]. However, p75 does not have a universal role in apoptosis, since many cell types that express p75 do not undergo programmed cell death. The generally accepted view of the newly found 'Jekyll and Hyde' nature of p75 is intriguing; p75 can promote apoptosis under some circumstances, while in other situations, it is a neuroprotective molecule [Carter and Lewin, 1997]. Recently, an intracellular protein which transduces the NGF/p75 death signal was identified and named NADE (p75NTR-associated cell death executor). This will be helpful for understanding the intracellular mechanism of apoptosis induced by p75 [Mukai et al., 2000].

To address the question of whether p75 is involved in the induction of RGC cell death in the developing rat, the topographic expression of p75 should be examined during the period of naturally occurring RGC cell death. Since it was difficult to demonstrate whether RGCs at this stage express p75 or not by means of simple morphological criteria, it is necessary to demonstrate the localization of p75 in retinae with retrogradely labeled RGCs. Most RGCs, if not all, have made synaptic contacts at the SC after birth, though we could not exclude the possibility that a few RGCs die before making a connection to the SC. A population of RGCs that die before making a connection would not be counted in this study, as the RGCs must have extended their axons to the SC before they can take up FG. The failure to detect p75 in retrogradely FG-labeled RGCs in retinal whole mounts and sections (fig. 3, 4) at different time points in this study suggested that p75 might not be expressed on RGCs. The p75-immunopositive staining which was reported previously on RGCs [Carmignoto et al., 1991] could, therefore, be on Müller cell processes surrounding the RGCs as previously reported in normal adult, injured and regenerating rat retinae [Hu et al., 1998, 1999].

Some previous studies have shown that p75 mRNA was detected in the developing retinal GCL with in situ hybridization using radioisotope-labeled probes [Koide et al., 1995; Ugolini et al., 1995]. By using Northern blot analysis, p75 mRNA was detected in the rat retina during the embryonic and postnatal periods [Takahashi et al., 1993]. Possible explanations for the discrepancy between our results and other previous observations are as follows: (1) the higher sensitivity of in situ hybridization for detecting p75 mRNA in RGCs in comparison to the detection of p75 receptor protein by immunocytochemistry; (2) the distribution of the silver grains developed from the radioisotope-labeled probes in in situ hybridization is too diffuse to localize p75 precisely at the cellular level in the GCL, especially considering the close relationship between RGCs and Müller cell processes; (3) identification of RGCs based only on morphological criteria was applied in previous studies without retrograde labeling of RGCs or confirmation with a double-staining method, which we routinely used in our series of studies [Hu et al., 1998, 1999] (present study); (4) no direct evidence has been found that the functional 'death domain' of the intracellular part of the p75 receptor protein is localized in RGCs, and (5) the probe used for p75 Northern blot analysis was 2.5 times longer (see Materials and Methods for detail) than in the previous study [Takahashi et al., 1993]. In general, the shorter the probe is, the less specific the hybridization will be. Other minor differences also exist, such as the total RNA extraction method (our Trizol commercially used reagents vs. traditional acid guanidinium thiocyanate-phenol-chloroform-extraction method) and the image analyzer used (ImageQuant program vs. other softwares previously used).

The DNA laddering confirmed the occurrence of apoptosis in the developing retinae (fig. 5). The maximum number of TUNEL-positive RGCs was found around PN1 (fig. 8). Some apoptotic cells located in the GCL were identified as RGCs by morphological criteria and FG prelabeling (fig. 6). However, we could not completely rule out the possibility that some apoptotic cells within the GCL in paraffin sections are displaced amacrine cells [Perry, 1981].

Since p75 mRNA expression is at a very low level during early development (from E14 to PN7) (fig. 1), and p75 presented neither on FG-prelabeled RGCs (fig. 3, 4) nor on TUNEL-positive cells (fig. 7), it is unlikely that p75 directly induces apoptosis of RGCs during naturally occurring cell death in the developing rat retina. Comparison of the numbers of apoptotic RGCs in both wild-type and p75 knock-out mice was also initially performed. There was no difference in the number of apoptotic RGCs in the retinae of wild-type and p75 knock-out mice at PN1 [unpubl. data]. This again suggests that p75 is not involved in RGC apoptosis.

However, p75 may be indirectly involved in the apoptosis of RGCs. In a rat model of light-induced retinal degeneration, Harada et al. [2000] demonstrated that the glial-neuronal cell interaction is important for the determination of photoreceptor apoptosis. Müller cells can alter basic fibroblast growth factor (bFGF) production in response to exogenous NGF and NT-3. bFGF is known to stimulate rat photoreceptor survival directly through FGF receptors and to prevent photoreceptor degeneration produced by constant light. NT-3 increases bFGF production via TrkC on Müller glial cells, which may rescue photoreceptors. In contrast, NGF reduces bFGF production via p75 expressed on Müller cells, which may lead to photoreceptor cell apoptosis. This could also happen to the RGCs during development. However, since the p75 transcript level is very low during the period of naturally occurring cell death in the rat retina (fig. 1), it is doubtful that the glial-neuronal cell interaction network could exert an apoptosis-determining function at that time.

In summary, RGCs undergo apoptosis during development, and the peak time of naturally occurring cell death is around PN1, when the p75 mRNA level is at its lowest. With a double immunohistochemistry method, we found that p75 was not expressed on RGCs but on processes around RGC bodies. There was no evidence in our study to support the notion that p75 induces RGC apoptosis directly. The major role of p75 in the retina might be in helping the Trks to mediate neurotrophic effects rather than exerting a proapoptotic effect [Hu et al., 1999].

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