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Effect of p75^{NTR} on the regulation of naturally occurring cell death and retinal ganglion cell number in the mouse eye

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Abstract

Neurotrophins induce neural cell survival and differentiation during retinal development and regeneration through the high-affinity tyrosine kinase (Trk) receptors. On the other hand, nerve growth factor (NGF) binding to the low-affinity neurotrophin receptor p75 (p75^{NTR}) might induce programmed cell death (PCD) in the early phase of retinal development. In the present study, we examined the retinal cell types that experience p75^{NTR}-induced PCD and identify them to be postmitotic retinal ganglion cells (RGCs). However, retinal morphology, RGC number, and BrdU-positive cell number in p75^{NTR} knockout (KO) mouse were normal after embryonic day 15 (E15). In chick retina, migratory RGCs express p75^{NTR}, whereas layered RGCs express the high-affinity NGF receptor TrkA, which may switch the pro-apoptotic signaling of p75^{NTR} into a neurotrophic one. In contrast to the chick model, migratory RGCs express TrkA, while stratified RGCs express p75^{NTR} in mouse retina. However, RGC number in TrkA KO mouse was also normal at birth. We next examined the expression of transforming growth factor β (TGFβ) receptor, which modulates chick RGC number in combination with p75^{NTR}, but was absent in mouse RGCs. p75^{NTR} and TrkA seem to be involved in the regulation of mouse RGC number in the early phase of retinal development, but the number may be later adjusted by other molecules. These results suggest the different mechanism of RGC number control between mouse and chick retina.

Keywords: Eye development; Retina; Programmed cell death; Neurotrophins; p75^{NTR}; TrkA; Retinal ganglion cell; Pax6; Mouse

Introduction

Neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), can support the survival and differentiation of neural cells during retinal development and regeneration (von Bartheld, 1998; Frade and Barde, 1999; Harada et al., 2005). Control of cell survival by neurotrophins is mediated by two types of transmembrane glycoproteins, the trk tyrosine kinase receptors (TrkA, TrkB and TrkC) and the neurotrophin receptor p75 (p75^{NTR}) (Parada et al., 1992; Barbacid, 1994; Kaplan and Stephens, 1994). Neurotrophins act in neural cell survival by activating trk tyrosine kinases, downstream of which a ras-dependent pathway leads to the activation of mitogen-activated protein (MAP) kinases (Klesse and Parada, 1999). Contrary to the action of neurotrophins on trk receptors, NGF binding to p75^{NTR} activates an intracellular pathway similar to that activated by death receptors such as tumor necrosis factor and Fas receptors, which involves ceramide production and thus induces apoptosis directly (Carter and Levin, 1997; Dechant and Barde, 1997). In fact, programmed cell death (PCD) in the early phase of retinal development is regulated by p75^{NTR} (Frade et al., 1996; Frade and Barde, 1999). p75^{NTR} is mainly expressed in retinal ganglion cells (RGCs) and Müller glial cells in the mature rodent retina (Vecino et al., 1998; Harada

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et al., 2000), however, the precise identity of the retinal cell types which experience $p75^{NTR}$-induced apoptosis during early development is not clear. Our present work demonstrates that $p75^{NTR}$ induces PCD in postmitotic RGCs.

Recent studies have proposed that stratified RGCs in chick retina themselves control RGC number by secreting NGF, which kills the incoming migratory RGCs via $p75^{NTR}$ on their surface (Gonzalez-Hoyuela et al., 2001; Frade et al., 1996). On the other hand, layered RGCs may survive the apoptotic effect of NGF by expressing the high-affinity TrkA receptor, which switches the pro-apoptotic signaling of $p75^{NTR}$ into a neurotrophic one (Gonzalez-Hoyuela et al., 2001). In the present study, we found the expression pattern in TrkA and $p75^{NTR}$ is inverted in mouse retina. To determine whether the different expression pattern reflects the different mechanism of RGC number control between chick and mouse retina, we also examined retinal morphology and RGC number in TrkA and $p75^{NTR}$ knockout (KO) mice.

Materials and methods

Animals

Experiments were performed using $p75^{NTR}$ KO mice (purchased from the Jackson Laboratory), TrkA KO mice (Liebl et al., 2000), and their littermates in accordance with the ARVO statement for the Use of Animals in Vision Research. Light intensity inside the cages ranged from 100 to 200 lx under 12 light:12 dark cycle. For developmental studies, mice were sacrificed at embryonic day 12 (E12), E13, E15, E17, postnatal day 0 (P0), P5, P10 and P40.

Genotyping of TrkA KO mice was carried out using the following PCR conditions: 94°C, 1 min; 55°C, 1 min; 72°C, 1 min; 34 cycles. The primers are: 5′-TGT ACG GCC ATA GAT AAG CAT-3′, 5′-TTG CAT AAC TGT GTA TTT CAC-3′, and 5′-CGC CTT CTT GAC GAG TTC TTC TG-3′. The size of the wild-type and mutant bands are ~160 bp and ~600 bp, respectively.

Histology

For E12, E13 and E15 mice, their heads were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.5% picric acid for overnight at 4°C and embedded in paraffin wax. They were sectioned transversely at 7 μm thickness, mounted and stained with hematoxylin and eosin. For P0, P5, P10, P20 and P40 mice, they were anesthetized with diethylether and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.5% picric acid at room temperature. The eyes were removed and postfixed in the same fixative for 2 h at 4°C and embedded in paraffin wax. The posterior part of the eyes was sectioned sagittaly at 7 μm thickness through the optic nerve, mounted and stained with hematoxylin and eosin.

TUNEL staining

Sections were incubated in 0.26 U/μl TdT in the supplied 1× buffer (Life Technologies, Carlsbad, CA), and 20 μM biotinylated-16-dUTP (Roche, Basel, Switzerland) for 60 min at 37°C. Sections were washed three times in phosphate buffered saline (PBS) (pH 7.4) and blocked for 30 min with 2% BSA in PBS (pH 7.4). The sections were then incubated with peroxidase conjugated streptavidin (Nichirei, Tokyo, Japan) for 30 min and visualized with DAB substrate kit (DAKO Corporation, Carpinteria, CA).

BrdU assay

Pregnant or P0 mice were pulsed with BrdU for 1 h. The dose of BrdU was 50 μg/g [body weight]. BrdU-treated mice were fixed and processed as described above. Sections were mounted on slides, heated in citric acid (0.01 M, pH 6.0) for antigen retrieval, and treated with 200 μg/ml pepsin and 2 M HCl. They were then incubated overnight with a mouse monoclonal antibody against BrdU (5 μg/ml, Roche), and visualized with DAKO Envision kit (DAKO).

Cell counts

The eyes were sectioned at 7 μm thickness through the optic nerve. The number of TUNEL- or BrdU-positive cells was counted from one ora serrata through the optic nerve to the other ora serrata. Six sections were examined at each time point.

Immunohistochemistry

For immunohistochemical staining, the sections were incubated with PBS containing 10% normal donkey serum for 30 min at room temperature. They were then incubated overnight with a rabbit polyclonal antibody against $p75^{NTR}$ (Promega, Madison, WI; 5.0 μg/ml), TrkA (Santa Cruz Biotechnology, Santa Cruz, CA; 1.0 μg/ml), a goat polyclonal antibody against Brn3b (Santa Cruz Biotechnology; 1.0 μg/ml), a mouse monoclonal antibody against calretinin (Chemicon, Temecula, CA; 1.0 μg/ml), calbindin (Sigma, Saint Louis, MO; 2.0 μg/ml), PKCα (Sigma; 2.0 μg/ml), glutamine synthetase (GS; Chemicon; 1.0 μg/ml), glial fibrillary acidic protein (GFAP; Progen, Heidelberg, Germany; 5.0 μg/ml), nestin (Developmental Studies Hybridoma Bank, Iowa City, IA; 1.0 μg/ml), Pax6 (Developmental Studies Hybridoma Bank; 35 μg/ml), TGFβRII (Santa Cruz Biotechnology; 1.0 μg/ml), and visualized with DAKO Envision kit (DAKO), Cy3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Groove, PA; 1000×), Cy2-conjugated donkey anti-goat IgG (Jackson Immunoresearch; 1000×) and FITC-conjugated donkey anti-mouse IgG (Jackson Immunoresearch; 200×). In some experiments, nuclear staining was performed with Hoechst 33342 (Molecular Probes, Eugene, OR; 10 μM) or 4,6-diamidino-2-phenylindole (DAPI; Wako, Osaka, Japan; 1000×).

Quantification of retinal cell apoptosis

Cell death was quantified by an ELISA (Roche) using a combination of antibodies recognizing histones and DNA, allowing the quantification of soluble nucleosomes in cell lysates (Harada et al., 2000). A mouse retina was homogenized in 100 μl PBS containing 1 mM PMSF and centrifuged at 15,000 × *g* for 10 min. A portion of the supernatant was used to quantify protein concentration, and the rest was processed. Absorbance values ranged between 0.1 and 1.0 and were normalized with respect to the values obtained with control retinas.

Statistical analysis

Data are presented as mean ± SEM except as noted. When statistical analysis was performed, one-factor ANOVA was used to estimate the significance of the results. Statistical significance was accepted at $P < 0.05$.

Results

Effect of $p75^{NTR}$ on programmed cell death in the developing retina

We first examined cell death in early stages of mouse retinal development using TUNEL staining from E12 to P0. At E12, TUNEL-positive nuclei were observed throughout the retina, and most of them were located near the optic nerve (Figs. 1A and C). To determine the role of $p75^{NTR}$ in cell death of the developing retina, we carried out similar experiments using $p75^{NTR}$ KO mice. Consistent with wild-type (WT) mice, TUNEL-positive nuclei were mostly located near the optic nerve, but the number seemed to be smaller (Figs. 1B and D). These results suggest a possibility that

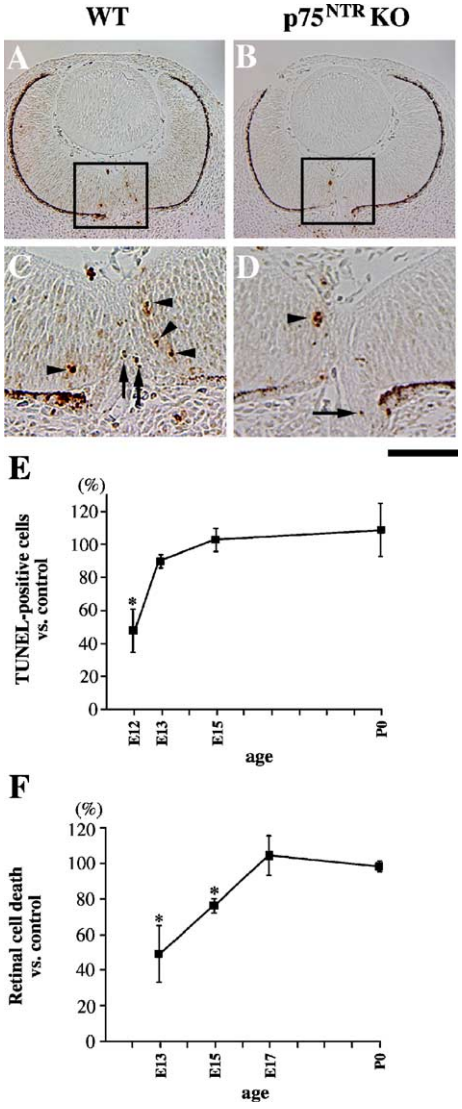

Fig. 1. Cell death in the developing retina of WT and p75^{NTR} KO mice. (A, B) TUNEL-positive nuclei in the retina of E12 WT (A) and p75^{NTR} KO (B) mice. TUNEL-positive cells are mainly observed in the central retina near the optic nerve head. (C, D) High magnification of the region included in the area from A and B, respectively. TUNEL-positive cells were observed in the neuroblast layer that is composed of poorly differentiated cells (arrowheads) and optic nerve (arrows). Note the decreased number of TUNEL-positive cells in p75^{NTR} KO mice. (E) Number of TUNEL-positive cells per 7 μm section of p75^{NTR} KO mice as a percentage of that of WT mice. The number of TUNEL-positive cells was counted from one ora serrata through the optic nerve to the other ora serrata. (F) Quantitative analysis of retinal cell apoptosis in p75^{NTR} KO mice as a percentage of that in WT mice. Apoptotic levels were quantified using an assay measuring soluble nucleosomes in retinal extracts. Retinal cell apoptosis in p75^{NTR} KO mice is decreased at E13 and E15, but normal after E17. In E and F, each data point represents the mean ± SEM of the values obtained from six independent experiments. *$P < 0.05$. Scale bar, 100 μm in (A, B) and 33 μm in (C, D).

p75^{NTR} in the embryonic retina might induce cell death (Frade and Barde, 1999). To assess this possibility, we counted the number of TUNEL-positive nuclei in the retinal sections that include the optic nerve in both strains. Compared with WT mice, the number of TUNEL-positive nuclei in p75^{NTR} KO mice was 48 ± 13% ($n = 6$) at E12 ($P < 0.05$) (Fig. 1E). However, the number was almost normal after E13. We next quantified programmed cell death in the whole retina using an ELISA method (Frade and Barde, 1999; Harada et al., 2000). In this sensitive method, reduced retinal cell death in p75^{NTR} KO mice was detected at E13 and E15, although it was normal after E17 (Fig. 1F). Thus, endogenous p75^{NTR} may induce programmed cell death (PCD) in early stages of mouse retinal development.

Effect of p75^{NTR} on retinal cell proliferation in the developing retina

To determine the role of p75^{NTR} in cell proliferation in early stages of mouse retinal development, we performed a BrdU assay for WT and p75^{NTR} KO mice from E12 to P0. At E12, consistent with WT mice (Fig. 2A), BrdU-positive cells were observed throughout the retina, but the number seemed to be smaller in p75^{NTR} KO mice (Fig. 2B). These results suggest a possibility that p75^{NTR} might increase the rate of cell proliferation in the embryonic retina. Therefore, we next counted the number of BrdU-positive cells in the retinal sections which include the optic nerve in both strains. Compared with WT mice, the number of BrdU-positive cells in p75^{NTR} KO mice was 82 ± 7% at E12, 85 ± 6% at E13, and 83 ± 6% at E15 ($n = 6$; $P < 0.05$), although it was almost normal at P0 (Fig. 2C). Together with the marked reduction

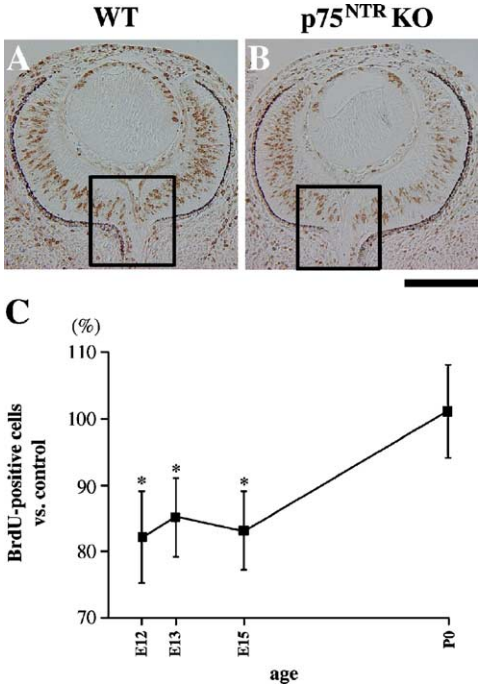

Fig. 2. Retinal cell proliferation in WT and p75^{NTR} KO mice. (A, B) BrdU-positive cells in the retina of E12 WT (A) and p75^{NTR} KO (B) mice. (C) Number of BrdU-positive cells per 7 μm section of p75^{NTR} KO mice as a percentage of that of WT mice. The number of BrdU-positive cells was counted from one ora serrata through the optic nerve to the other ora serrata. BrdU-positive cells in p75^{NTR} KO mice are decreased at E12, E13, and E15, especially near the optic nerve head (enclosed area in A and B), but normal at birth. Each data point represents the mean ± SEM of the values obtained from six independent experiments. *$P < 0.05$. Scale bar, 100 μm.

near the optic nerve (enclosed area in Figs. 2A and B), endogenous p75^{NTR} may induce PCD (Fig. 1) and cell proliferation in early stages of mouse retinal development.

Effect of p75^{NTR} on retinal morphology

Since p75^{NTR} may increase both PCD and proliferation in the developing retina, we next examined whether the development of the retina is perturbed in p75^{NTR} KO mice. Figs. 3A–F illustrate the development of embryonic eye in WT and p75^{NTR} KO littermates. The size and construction of the eye in p75^{NTR} KO mice (Figs. 3B, D and F) seemed to be normal including the region near the optic nerve. Figs. 3G–N illustrates representative steps in postnatal retinal development. In WT retina, the structure of the ganglion cell layer (GCL) was clearly visible at P0 (Fig. 3G). GCL was composed of multiple layers at P0 and P5 (Figs. 3G and I), but its thickness decreased and it becomes a monolayer by P10 (Fig. 3K). Other than the

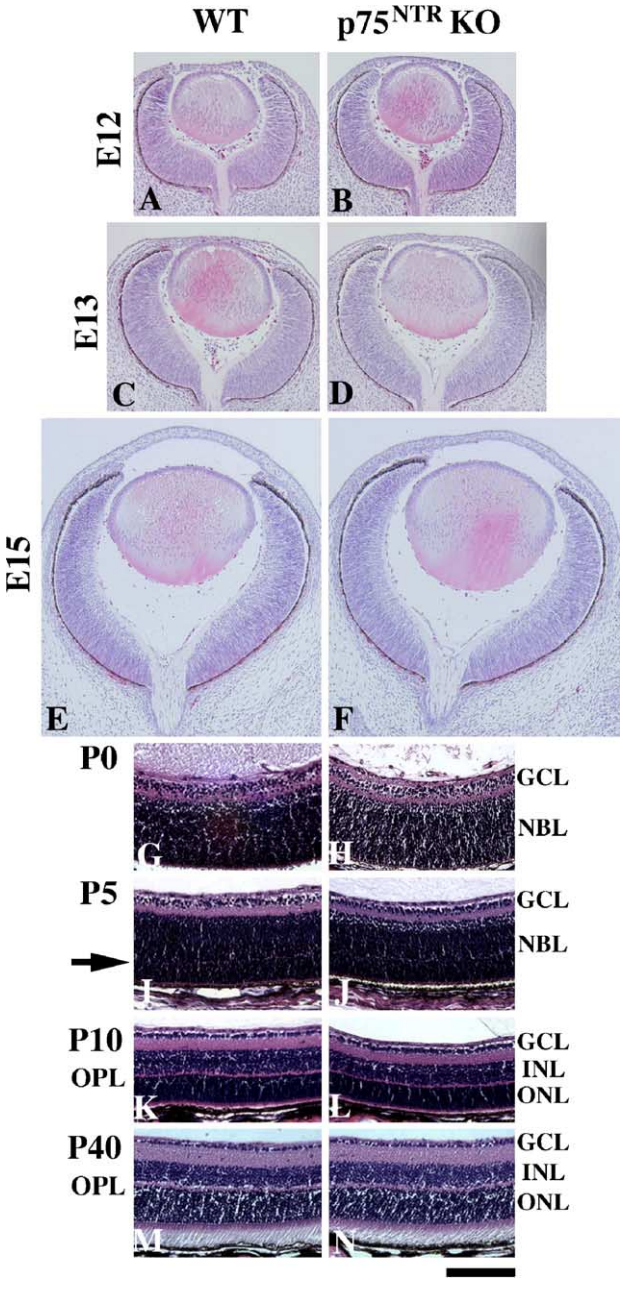

Fig. 3. Embryonic and postnatal eye development in p75^{NTR} KO mice. Hematoxylin and eosin staining of retinal sections at E12 (A, B), E13 (C, D), E15 (E, F), P0 (G, H), P5 (I, J), P10 (K, L), and P40 (M, N) in WT (A, C, E, G, I, K, M) and p75^{NTR} KO (B, D, F, H, J, L, N) littermates. Note the normal development in p75^{NTR} KO mice. GCL, ganglion cell layer; NBL, neuroblast layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar, 100 μm.

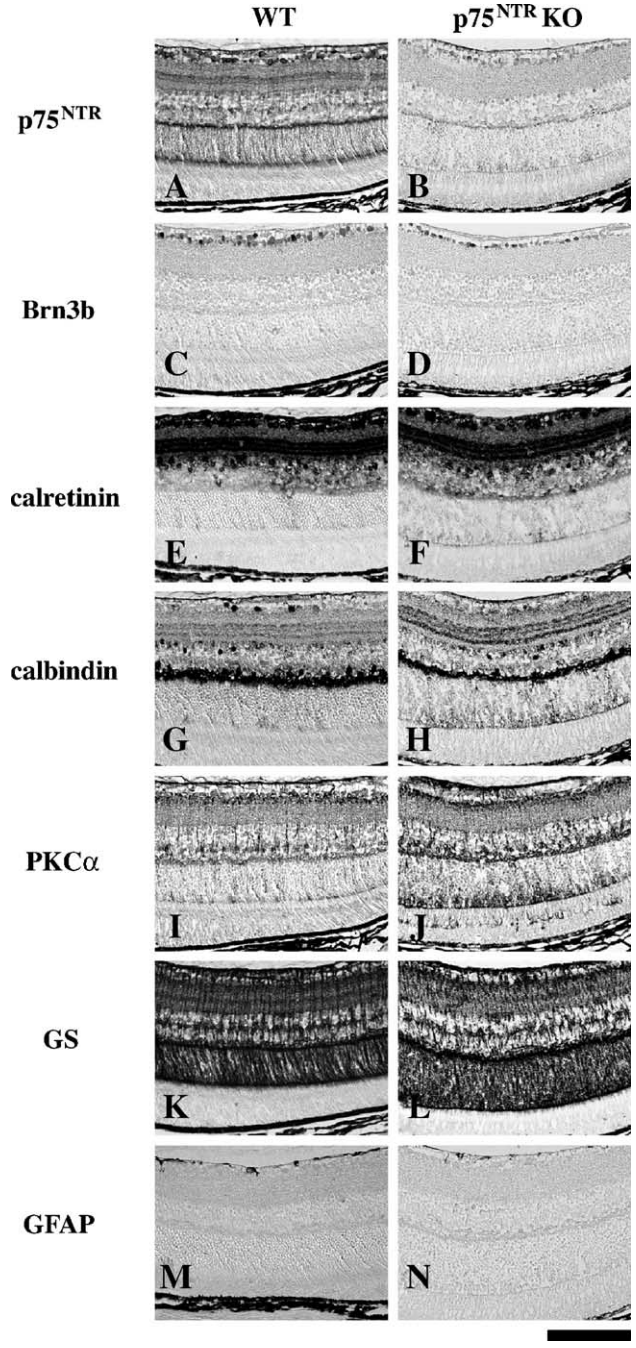

Fig. 4. Expression of cell type specific markers in WT and p75^{NTR} KO P40 mice retina. Immunohistochemical analysis of p75^{NTR} (A, B), Brn3b (C, D), calretinin (E, F), calbindin (G, H), PKCα (I, J), glutamine synthetase (GS; K, L) and GFAP (M, N) in WT (A, C, E, G, I, K, M) and p75^{NTR} KO (B, D, F, H, J, L, N) littermates. Scale bar, 100 μm.

GCL, only the neuroblast layer (NBL), which is composed of poorly differentiated cells, was visible at P0 (Fig. 3G). The NBL is segregated into the inner nuclear layer (INL) and the outer nuclear layer (ONL), which is composed of photoreceptor nuclei, by the outer plexiform layer (OPL). The OPL starts to form at P5 (Fig. 3I, arrow) and is visible as a gap between the INL and the ONL (Figs. 3K and M). This developmental sequence occurred in their $p75^{NTR}$ KO littermates, and the time course was normal (Figs. 3H, J, L and N). We further examined WT and $p75^{NTR}$ KO mouse retinas with various cell-type specific markers (Fig. 4). The expression pattern of Brn3b (ganglion cells; Figs. 4C, D), calretinin (ganglion and amacrine cells; Figs. 4E, F), calbindin (horizontal cells; Figs. 4G, H), PKCα (bipolar cells; Figs. 4I, J), glutamine synthetase (GS; Müller cells; Figs. 4K, L), and glial fibrillary acidic protein (GFAP; astrocytes; Figs. 4M, N) showed no remarkable change between WT and $p75^{NTR}$ KO mouse retinas. Thus, the absence of $p75^{NTR}$ signaling did not seem to be involved in the differentiation and migration of retinal cells and their assembly into defined layers.

$p75^{NTR}$ expression in the developing retina

We next examined the expression of $p75^{NTR}$ in the developing retina. At E15, $p75^{NTR}$-like immunoreactivity was observed in the GCL throughout the retina and around the optic nerve head (red in Fig. 5A). Since retinal cell apoptosis was observed around the optic nerve head (arrows in Figs. 1C and D), $p75^{NTR}$ might be expressed in glial cells within the optic nerve. To determine this possibility, we carried out double-labeling immunohistochemistry using $p75^{NTR}$ and GFAP antibodies. GFAP-positive cells were not detected at E15 (Fig. 5A), but observed in optic nerve at P0 (green in Fig. 5B) and some cells are double-labeled with $p75^{NTR}$. Thus, $p75^{NTR}$ may induce PCD in some glial cells in the optic nerve after birth, but not in the early stages of mouse retinal development.

Since TUNEL-positive cells were also observed in the NBL (arrowheads in Figs. 1C and D), other cell types that may experience programmed cell death are retinal stem and/or progenitor cells. To determine this possibility, we carried out double-labeling immunohistochemistry using $p75^{NTR}$ and nestin antibodies. At E12, nestin-positive cells were observed in the subretinal space and vitreous body, and many cells were double-labeled with $p75^{NTR}$ (yellow in Fig. 5C), however, they were not detected in the neural retina. Although the number of nestin-positive cells was smaller, similar results were observed at E15 (data not shown). The number of nestin-positive cells was normal in $p75^{NTR}$ KO mice (Fig. 5D). We next examined whether $p75^{NTR}$ is co-expressed with a nuclear marker of both ganglion and amacrine cells, Pax6 (Marquardt et al., 2001) and found many $p75^{NTR}$/Pax6 double positive cells (Fig. 5E). Since $p75^{NTR}$ is mainly expressed in the immature GCL at E15 (Figs. 5A and E), we next employed Brn3b antibody that is a marker of the postmitotic retinal ganglion cells (RGCs), and found that many $p75^{NTR}$-positive cells are double-labeled with Brn3b (Fig. 5F). Taken together, these results suggest that $p75^{NTR}$ induces PCD in postmitotic RGCs.

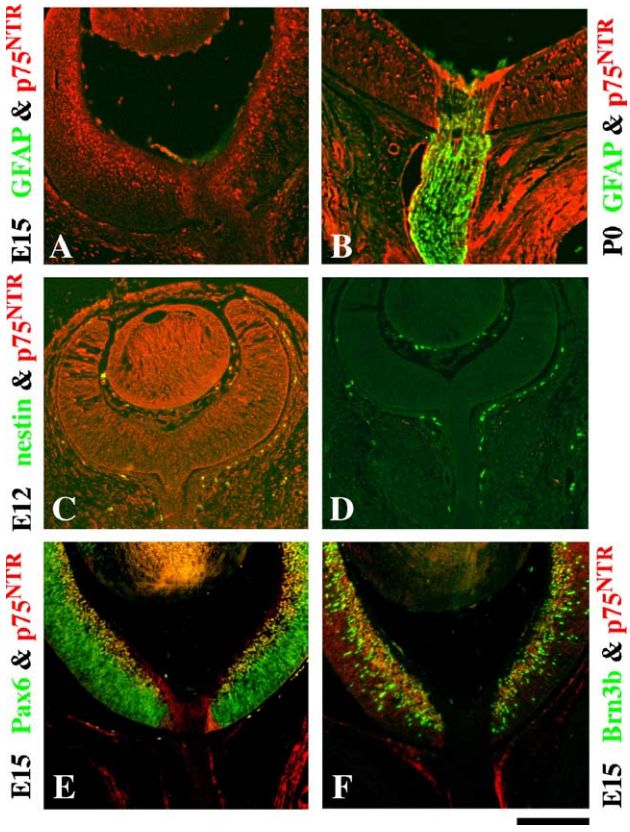

Fig. 5. Expression of GFAP, nestin, Pax6, and Brn3b in WT and $p75^{NTR}$ KO mice retina. (A, B) Immunohistochemical analysis of E15 (A) and P0 (B) mouse retina using the antibodies for GFAP (green) and $p75^{NTR}$ (red). Some cells are double-labeled with $p75^{NTR}$ (yellow) near the optic nerve head in (B), but not in the neural retina. (C, D) Immunohistochemical analysis of E12 mouse retina using the antibodies for nestin (green) and $p75^{NTR}$ (red) in WT (C) and $p75^{NTR}$ KO (D) mice. Many nestin-positive cells are double-labeled with $p75^{NTR}$ (yellow) in (C), but they are absent in the neural retina. Number and distribution of nestin-positive cells are normal in $p75^{NTR}$ KO mice (D). (E, F) Immunohistochemical analysis of E15 mouse retina using the antibodies for Pax6 (E) or Brn3b (F) (green) and $p75^{NTR}$ (red). Many cells in the inner part of the neural retina are double-labeled with $p75^{NTR}$ (yellow in E and F). Scale bar, 100 μm in (A, B) and 90 μm in (C–F).

Expression pattern of $p75^{NTR}$, TrkA and TGFβ receptor in the mouse retina

The initial phase of RGC death affects postmitotic cells during their migration from the ventricular to the vitreal surface of the retina (Frade et al., 1997). In chick retina, stratified RGCs may reduce the number of migratory RGC via $p75^{NTR}$ on their surface (Gonzalez-Hoyuela et al., 2001; Frade et al., 1996). On the other hand, layered RGCs expressing the high-affinity TrkA receptor switches the pro-apoptotic signaling of $p75^{NTR}$ into a neurotrophic one, thus ensuring RGC survival (Gonzalez-Hoyuela et al., 2001). Since we could detect Brn3b-positive migratory RGCs in the NBL at E15 (green in Fig. 5F), we examined the expression of $p75^{NTR}$ and TrkA receptors in E15 retina. $p75^{NTR}$-like immunoreactivity was observed in the inner retina including the GCL (Figs. 6A and C), but TrkA-like

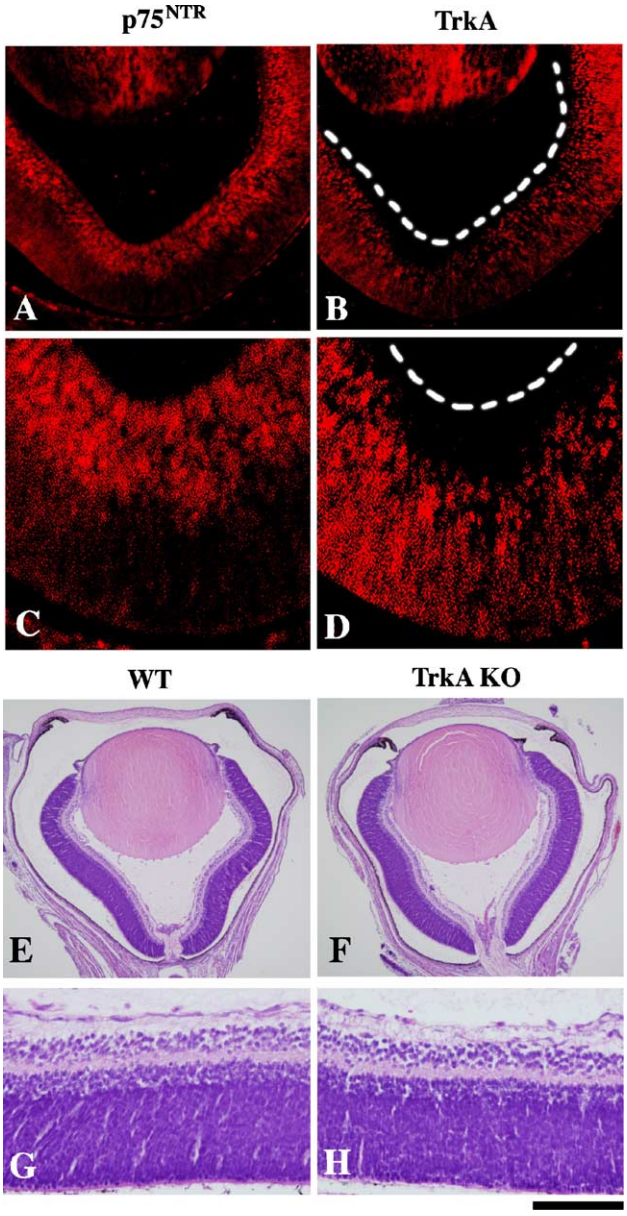

Fig. 6. Expression of p75^{NTR} and TrkA in the developing mouse retina. (A–D) Immunohistochemical analysis of E15 mouse retina using the antibody for p75^{NTR} (A, C) or TrkA (B, D). p75^{NTR}-like immunoreactivity was observed in the inner retina including the GCL, but TrkA-like immunoreactivity was mainly observed in the outer retina. (E–H) Hematoxylin and eosin staining of eye sections at P0 in WT (E, G) and TrkA KO (F, H) littermates. Scale bar, 100 μm in (A, B, G, H), 33 μm in (C, D) and 500 μm in (E, F).

immunoreactivity was mainly observed in the outer retina (Figs. 6B and D). We also determined that many p75^{NTR}-positive cells are Brn3b-positive RGCs (Figs. 7A–C), and some p75^{NTR}- and Brn3b-positive cells in the inner part of the neural retina showed condensed or fragmented nuclei, indicating that these cells were undergoing apoptosis (arrowheads in Fig. 7D). These results are partially consistent with those of previous reports (Gonzalez-Hoyuela et al., 2001; Frade et al., 1996), but the distribution of p75^{NTR} and TrkA are inversed when compared to the chick model of RGC development. To determine the effect of inverted expression pattern of TrkA during RGC migration, we next examined TrkA KO mice at P0 because they die soon after birth.

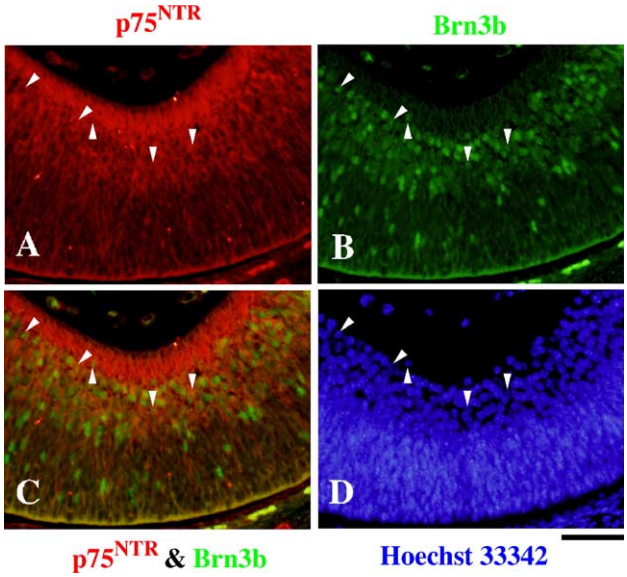

Fig. 7. Expression of p75^{NTR} and cell death in the developing mouse retina. (A–C) Immunohistochemical analysis of E15 mouse retina using the antibody for p75^{NTR} (red in A) and Brn3b (green in B). Many cells are double-labeled in the inner part of the neural retina (yellow in C). (D) Nuclear staining with Hoechst 33342 of the same section. Note that some p75^{NTR}- and Brn3b-positive postmitotic RGCs appeared to be apoptotic (arrowheads). Scale bar, 50 μm.

However, the size of the eyes and retinal structure were normal in TrkA KO mice (Figs. 6E–H).

Our results suggest a possibility that p75^{NTR} and TrkA may regulate PCD in postmitotic RGCs in early stages of mouse retinal development. However, retinal development was almost normal in both p75^{NTR} and TrkA KO mice (Figs. 3, 4, and 6). Thus, other molecules may compensate for the function of p75^{NTR} and TrkA. One possible molecule is TGFβ receptor II because it is coexpressed with p75^{NTR}, and both TGFβ and NGF are required to regulate cell death in the chick retina in vivo (Dünker et al., 2001). We carried out immunohistochemistry for TGFβ receptor II in the mouse retina, but their immunoreactivity was not detected in neural retina at both E15

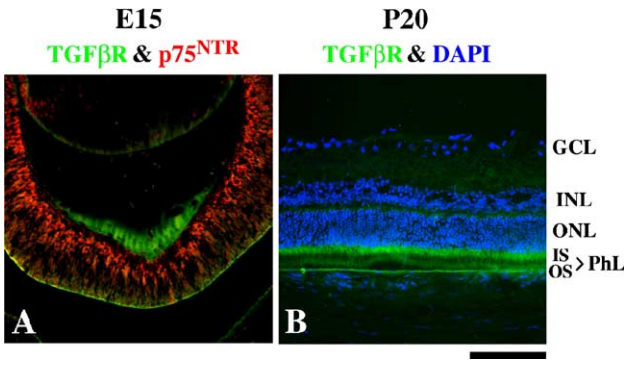

Fig. 8. Expression of TGFβ receptor II in the mouse retina. Immunohistochemical analysis of E15 (A) and P20 (B) mouse retina using the antibodies for TGFβ receptor II (TGFβR; green) and p75^{NTR} (red). TGFβ receptor II-like immunoreactivity is observed in the photoreceptor cell layer (B), but not in the neural retina including retinal ganglion cells (A, B). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PhL, photoreceptor layer; IS, inner segment; OS, outer segment. Scale bar, 100 μm.

and P20 (Fig. 8). These results also suggest a different mechanism for postmitotic RGC regulation in mouse and chick retina.

Discussion

In the present study, we demonstrate that low-affinity neurotrophin receptor $p75^{NTR}$ induces programmed cell death (PCD) during mouse retinal development (Fig. 1; Frade and Barde, 1999). However, the apoptotic level (Fig. 1) and retinal morphology (Figs. 3 and 4) in $p75^{NTR}$ KO mouse is normal after E15. Consistently, BrdU-positive cell number is significantly decreased before E15, but normal at P0 (Fig. 2). Frade (2000) demonstrated that unscheduled re-entry of postmitotic neurons into the cell cycle precedes neural cell death in $p75^{NTR}$ expressing chick retinal cells. Thus, decreased BrdU incorporation in the $p75^{NTR}$ KO mouse may correspond to a decreased rate of both retinal proliferation and cell death. A previous study reported that the BrdU-positive cell number is normal in subplate neurons in $p75^{NTR}$ KO mouse after E18.5 (McQuillen et al., 2002). The differences seen in the present report might reflect analysis of different region and different time points examined.

Another important point is the identification of cell types that experience $p75^{NTR}$-induced apoptosis. In the present study, we identified that the cell type susceptible to $p75^{NTR}$-induced death is the postmitotic RGC (Fig. 7). In chick retina, stratified RGCs secrete NGF and kill incoming migratory RGCs via $p75^{NTR}$ on their surface, while layered RGCs survive the apoptotic effect by expressing the high-affinity TrkA receptor (Gonzalez-Hoyuela et al., 2001). TrkA is essential for the survival and differentiation of sensory neurons, and is regulated by Brn3a POU-domain transcription factor (Ma et al., 2003), which is capable of promoting RGC development (Liu et al., 2000). Interestingly, our results in mouse retina differ from the chick model. Migrating RGCs in the NBL express TrkA, while stratified RGCs express $p75^{NTR}$ in the immature GCL (Fig. 6). However, RGC number was normal in both $p75^{NTR}$ and TrkA KO mouse (Figs. 3, 4, and 6). Our results suggest that $p75^{NTR}$ regulates PCD in only the early phase of retinal development, and retinal cell number and morphology might be adjusted in the late period largely corresponding to the phase of target-dependent PCD (Cook et al., 1998; Cellerino et al., 2000). Possible molecules that control RGC number are TGFβ and sonic hedgehog (Shh) in chick retina (Dünker et al., 2001; Zhang and Yang, 2001). However, the mechanism might be different between chick and mouse. In mouse retina, TGFβ receptor is absent from neural retina (Fig. 8). In addition, Shh signaling from RGCs has no effect on RGC number although it is required for optic disc and stalk neuroepithelial cell development (Wang et al., 2002a; Dakubo et al., 2003). Other possible regulators of mouse RGC number are trophic factors other than NGF (von Bartheld, 1998; Harada et al., 2005). In addition to TrkA, mouse RGCs express TrkB that is a high-affinity receptor for BDNF and NT-4/5 (Cellerino and Kohler, 1997; Llamosas et al., 1997; Rohrer et al., 1999). Consistently, BDNF or NT-4/5 injected into the superior colliculus (SC) promotes the survival of neonatal RGCs (Cui and Harvey, 1995; Ma et al., 1998). Thus, a balance between the neuroprotective effect of BDNF or NT-4/5 and the killing activity of NGF may affect the relative expression levels of trk receptors and $p75^{NTR}$, which determines the fate of RGCs.

Recent studies have shown that trophic factors such as BDNF, ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), or glial cell line-derived neurotrophic factor (GDNF) increase RGC survival and regeneration (Mey and Thanos, 1993; Cohen et al., 1994; Mansour-Robaey et al., 1994; Unoki and LaVail, 1994; Hammes et al., 1995; Bosco and Linden, 1999; Di Polo et al., 1998; Yan et al., 1999; Peterson et al., 2000; Koeberle and Ball, 2002). We previously showed that these factors, in addition to direct neuroprotection, can alter secondary trophic factor production in retina-specific Müller glial cells, which indirectly regulate neural cell survival (Harada et al., 2000, 2002, 2003). Consistently, intraocular injection of trophic factors induces the phosphorylated form of extracellular receptor kinase (pERK) or c-*fos* mainly in Müller cells (Wahlin et al., 2000, 2001). Thus, trophic factor receptors that are expressed in RGCs and/or Müller cells might protect stratified RGCs from $p75^{NTR}$-induced cell death, and play an important role for RGC number control. We are planning to examine the effect of various trophic factors and their receptors during retinal development in region-specific conditional KO mice (He et al., 2004; Luikart et al., 2005). Another possibility is an involvement of adhesion molecules in stratified RGCs (Honjo et al., 2000; Rattner et al., 2001; Xu et al., 2002). For example, cadherin is essential for the structural integrity of the outer segment and for photoreceptor survival. Since cadherin is expressed in RGCs, it might be also involved in the regulation of RGC survival and the process of RGC maturation.

In conclusion, our data show that $p75^{NTR}$ controls early phase of PCD in postmitotic RGCs in mouse retina. Recent studies have shown various biological aspects of $p75^{NTR}$. For example, $p75^{NTR}$ might be involved in photoreceptor degeneration in the mature retina (Harada et al., 2000, 2002; Bringmann and Reichenbach, 2001; Mitamura et al., 2005). In addition, $p75^{NTR}$ may interact with Nogo-66 receptor as a co-receptor for Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp), which lead to neurite outgrowth inhibition (Wang et al., 2002b). These findings may be of clinical relevance if these molecules are to be used therapeutically in the treatment of human retinal and optic nerve diseases that otherwise lead to severe visual impairment. For example, inhibiting biological activity of $p75^{NTR}$ by specific blocker, antisense oligonucleotide, or neutralizing antibodies may be useful for neural cell protection and regeneration (Harada et al., 2000, 2002; Yamashita and Tohyama, 2003). On the other hand, it is still controversial whether $p75^{NTR}$ is expressed in mature RGCs in rat retina (Hu et al., 1998; Ding et al., 2001). Furthermore, in other situations, $p75^{NTR}$ may be rather a neuroprotective molecule (Carter and Lewin, 1997) and necessary for myelin regeneration (Cosgaya et al., 2002). Thus, it is apparent that we need to solve these basic problems and reveal the precise role of $p75^{NTR}$ and its related molecules during retinal development and degeneration.

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