Molecular Vision 2007; 13:1311-8 <http://www.molvis.org/molvis/v13/a143/>
Received 2 February 2007 | Accepted 8 July 2007 | Published 25 July 2007

Comparison of expression profile of neurotrophins and their receptors in primary and transformed rat retinal ganglion cells

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Purpose: To determine the expression profile of neurotrophins and their receptors in cultured primary rat retinal ganglion cells (RGCs) and the transformed RGC-5 cells.
Methods: Confocal microscopic immunocytochemistry with double fluorescent labeling with thy-1 as a marker for RGCs was used to demonstrate expression of neurotrophins and their receptors. An enzyme-linked immunosorbent assay (ELISA) was used to detect secretion of neurotrophins by RGC-5 cells.
Results: Primary RGCs and RGC-5 cells expressed brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrohin-3 (NT3), neurotrophin-4 (NT4), and receptors TrkA, p75, with low levels of TrkB. However, minimal if any, expression of TrkC was observed in these cells. RGC-5 cells also secreted NT3 (1311±21 pg/ml), BDNF (92±9 pg/ml), NGF (86±7 pg/ml), and NT4 (21±1 pg/ml) into the cultured media.
Conclusions: These results demonstrated that neurotrophins and TrkA, p75 with low levels of TrkB receptors are expressed by RGCs. Specific neurotrophins acting through TrkA, TrkB, and p75 receptors within the RGCs may be involved in the survival and apoptosis of the RGCs in various retinopathies, such as glaucoma.

Neurotrophins are a family of proteins that promote the development, survival, and differentiation of neurons. Four members of the neurotrophin family have been identified in mammals and include nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4) [1]. Neurotrophins bind with high affinity to a class of tyrosine kinase receptors known as Trk receptors. The TrkA receptor has been shown to be the signaling receptor for NGF [2,3]. Other Trk receptors include TrkB, which binds BDNF and NT4, and TrkC, the signaling receptor for NT3 [1]. Neurotrophin receptor, p75, is a member of the tumor necrosis factor receptor family that includes TNFR-I, TNFR-II and the Fas antigen. The p75 receptor binds to all four neurotrophins [4].

Glaucoma triggers selective loss of RGCs [5-7]. The mechanism of this pathological change is yet to be elucidated. However, it has been proposed that elevated intraocular pressure in glaucoma results in obstruction of axonal transport at the optic nerve head, blocking the retrograde transport of neurotrophins to the cell soma [5,8-10]. Neurotrophic factor deprivation is thus a potential cause of RGC apoptosis in glaucoma.

This hypothesis is further supported by numerous studies indicating that these trophic factors not only improve the survival and neurite outgrowth of RGCs in culture [11-15], but also significantly reduce axotomy-induced in vivo damage of the optic nerve and RGC [16-22], as well as stimulate the growth of neurites from regenerating RGCs [18,23,24]. For example, a single intravitreal injection of BDNF prevents the death of the axotomized RGCs when administered at any time during the first five days after injury [18]. In contrast with the loss of nearly half of the axotomized RGC in the untreated retinas, virtually all RGCs were present one week after a single injection of BDNF on Day 0.

In spite of the importance of neurotrophins in the maintenance of the health of RGCs in retina and the protection of RGCs from insult-induced apoptosis, there are only a limited number of reports examining the expression profile of neurotrophins in RGCs, either in vivo or in vitro. For example, RGCs have been shown to express BDNF, NT3, NT4 and CNTF [5,25-28]. The distribution of other neurotrophins and their receptors in the retinal ganglion cells are not well documented. Therefore, the purpose of this descriptive but important study was to investigate the expression profile of neurotrophins and compare it to an established transformed rat RGC line, RGC-5, by confocal immunofluorescence microscopy using Thy-1 as an immunocytochemical specific marker to identify retinal ganglion cells.

METHODS

Culture of RGC-5 cells: RGC-5 cells were maintained on 12-mm glass coverslips in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 100 units/ml of penicillin and 100 µg/ml of streptomycin (Sigma, St Louis, MO) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C [14]. The cells had a doubling time of about 20 h and were passaged by trypsinization every three to four days. RGC5 cells of passages 10-20 were used in these studies.

Isolation and culture of adult rat retinal ganglion cells: Adult Sprague-Dawley rats were euthanized by CO_2 asphyxi-

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ation. All the chemicals for adult RGCs primary cultures were purchased from Sigma unless stated otherwise. Their eyes were enucleated and retina isolated in the Neurobasal/B27 medium (Gibco/InVitrogen, Carlsbad, CA). Retinal cells were dissociated by combining up to 20 retinas with 5 ml of papain solution, containing 10 mg papain, 2 mg DL-cysteine, and 2 mg bovine serum albumin in 5 ml of Neurobasal/B27 medium for 25 min at 37 °C, then washed 3 times with 5 ml RGC culture Neurobasal medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM pyruvate (Gibco/InVitrogen), 2 mM glutamine (Gibco/InVitrogen), 5 μg/ml insulin, 100 μg/ml transferrin, 100 μg/ml bovine serum albumin, 60 ng/ml progesterone, 16 μg/ml putrescine, 40 ng/ml sodium selenite, 40 ng/ml thyroxine, 40 ng/ml tri-iodothyronine, 50 ng/ml brain-derived neurotrophic factor (Biosource, Camarillo, CA), 10 ng/ml ciliary neurotrophic factor (Biosource), 10 ng/ml basic fibroblast growth factor (Biosource), 5 μM forskolin, and 1% fetal calf serum (Atlas Biologicals, Fort Collins, CO). Retinal pieces were triturated by passing through a fire-polished disposable pipette several times until cells were dispersed. Cell suspension was placed onto poly-D-lysine- and laminin-coated 8-well chambered culture slides (Becton Dickinson, San Jose, CA). The cells were then cultured for three days in 95% air/5% CO_2 at 37 °C. The Thy-1 positive cells were established to be negative for other retinal markers such as arrestin for photoreceptors; GFAP for astroglia and Muller cells; ED1 for microglia; and protein kinase C-α for rod bipolar cells [29].

Confocal microscopy: Primary and transformed rat RGCs were fixed in 4% paraformaldehyde, and rinsed in phosphate buffered saline (PBS; pH 7.4). The sections and cells were then immersed in a 0.1% Triton X-100 solution for 30 min and rinsed in PBS at least three times for 5 min each. Samples were then treated with a 0.05M solution of glycine for 15 min to eliminate free aldehydes. The cells were subsequently incubated in a blocking solution of 5% BSA and 10% normal serum of second antibody host animal in PBS for 15 min, followed by an overnight incubation at 4 °C with primary antibodies for BDNF, NGF, NT3, NT4, TrkA, TrkB, TrkC, p75, and Thy-1 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted to 1 μg/ml in a solution of 1% BSA and 1% normal serum of secondary antibody host animal in PBS. For controls, the primary antibody was omitted and replaced by goat IgG in the same concentration as the primary antibody. After the incubation, the study samples were rinsed four times in PBS and incubated with the secondary antibody coupled to Alexa red 633 or Alexa green 488 (Molecular Probes, Inc.) diluted 1:200 in buffer without normal serum for 1 h at room temperature. The cells were rinsed in PBS, mounted with antifade mounting media, and examined with a Zeiss LSM410 confocal microscope with a 40X water immersion objective (numerical aperture 1.2). Samples were excited with 488 nm (for Alexa 488) and 633 nm (for Alexa 633), fluorescent emission examined at 510-525 and 645-665 nm, respectively. The various antibodies used these studies were tested for their specificity by western blotting [30,31].

Enzyme-linked immunosorbent assays: RGC-5 cells were cultured to 75% confluence, then serum-deprived for 24 h. The conditioned media was collected and neurotrophins secreted by RGC-5 cells were determined by ELISA (Promega Corp., Madison, WI) according to the supplier's instructions.

RESULTS

Immunocytochemical localization of Thy-1: By confocal fluorescent microscopy, primary cultured adult rat RGCs were shown to express Thy-1 in the cell soma and neurites (Figure 1A). Similarly, RGC-5 cells also showed positive labeling for Thy-1 (Figure 1D). Since in the retina, only the RGCs were reported to express Thy-1 [32-34], these results demonstrate that the cultured RGCs, both primary and transformed cells, were indeed RGCs and they retained the Thy-1-positive phenotype.

Immunolocalization of BDNF: Both primary adult rat RGCs (Figure 1B) and RGC-5 cells (Figure 1E) had an intense BDNF immunoreactivity. Similar to Thy-1, BDNF was also detected in both the cell soma and neurites. Merged images of Thy-1 and BDNF immunofluorescence clearly showed that BDNF and Thy-1 were co-expressed in the same cells in primary RGCs as well as the transformed RGC-5 cells (Figure 1C,F).

Immunolocalization of NGF, NT3, and NT4: Both the cultured primary adult rat RGCs and transformed RGC-5 cells were positively labeled for NGF, NT3, and NT4 with their expression in both cell soma and neurites (Figure 2). (To avoid redundancy, only merged images of Thy-1 with these neurotrophins and the neurotrophin receptors are shown.) As expected, the IgG control produced no immunostaining in the cells (Figure 2A,E).

Immunolocalization of p75, TrkA, TrkB, and TrkC: Neurotrophin receptors, p75 and TrkA, were detected in cultured primary adult rat RGCs (Figure 3A,B), both in the cell bodies and neurites. Similar distribution patterns were demonstrated in the transformed RGC-5 cells as p75 and TrkA were localized in the cell soma and the neurites (Figure 3E,F). In contrast, cultured rat RGCs and RGC-5 cells were only weakly labeled for TrkB (Figure 3C,G), while TrkC receptor was undetectable (Figure 3D,H) as only red immunofluorescence for thy-1 was detected.

Secretion BDNF, NGF, NT3, and NT4 by RGC-5 cells: The RGC-5 cells were found to secrete neurotrophins. Their levels in conditioned medium of RGC-5 cells following a 24 h incubation were highest for NT3 (1311±21 pg/ml), followed by BDNF (92±9 pg/ml), and NGF (86±7 pg/ml; Figure 4). The levels of secreted NT4 were the lowest among the neurotrophins (21±1 pg/ml). These results demonstrate that RGC-5 cells synthesize and secrete neurotrophins in vitro.

DISCUSSION

Retinal ganglion cells play a key role in integrating visual information and relaying it to the visual centers of the brain via the optic nerve. The health of these cells depends on the availability of various neurotrophic factors. A number of studies have shown that neurotrophins promote survival of injured RGCs and other retinal neurons [35,36]. Exogenous BDNF in the chick embryo resulted in an increase in the number of sur-

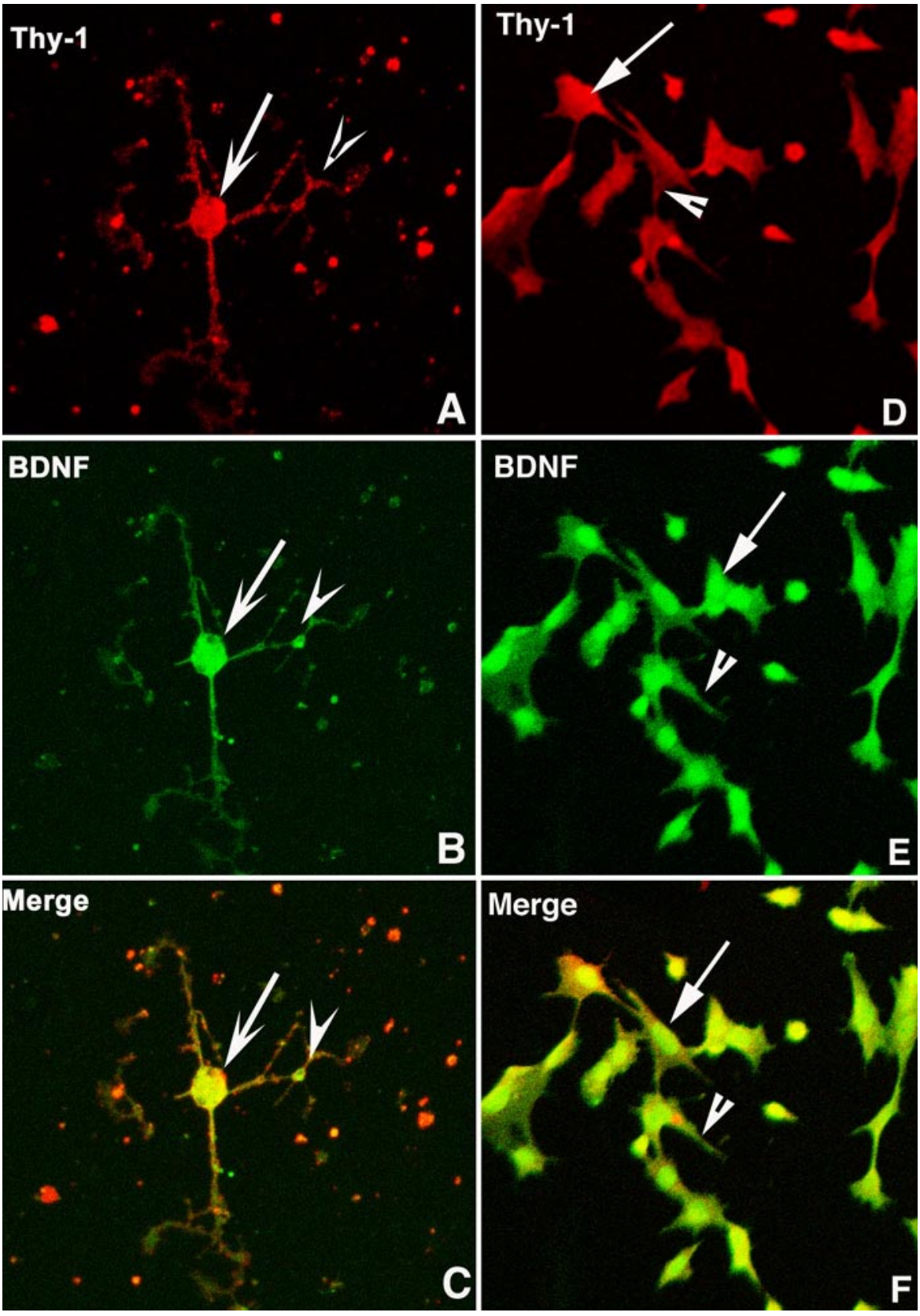

Figure 1. Confocal immunolocalization of brain-derived neurotrophic factor. Confocal immunocytochemical localization of Thy-1 (red) and brain-derived neurotrophic factor (BDNF; green) in primary culture of adult rat retinal ganglion cells (RGCs; **A** and **B**, respectively) and transformed RGC-5 cells (**D** and **E**, respectively). The merged images of these same cells are shown for primary (**C**) and transformed cells (**F**). BDNF and Thy-1 are co-expressed by both cells and are present in the soma (arrow) and the neurites (arrowhead).

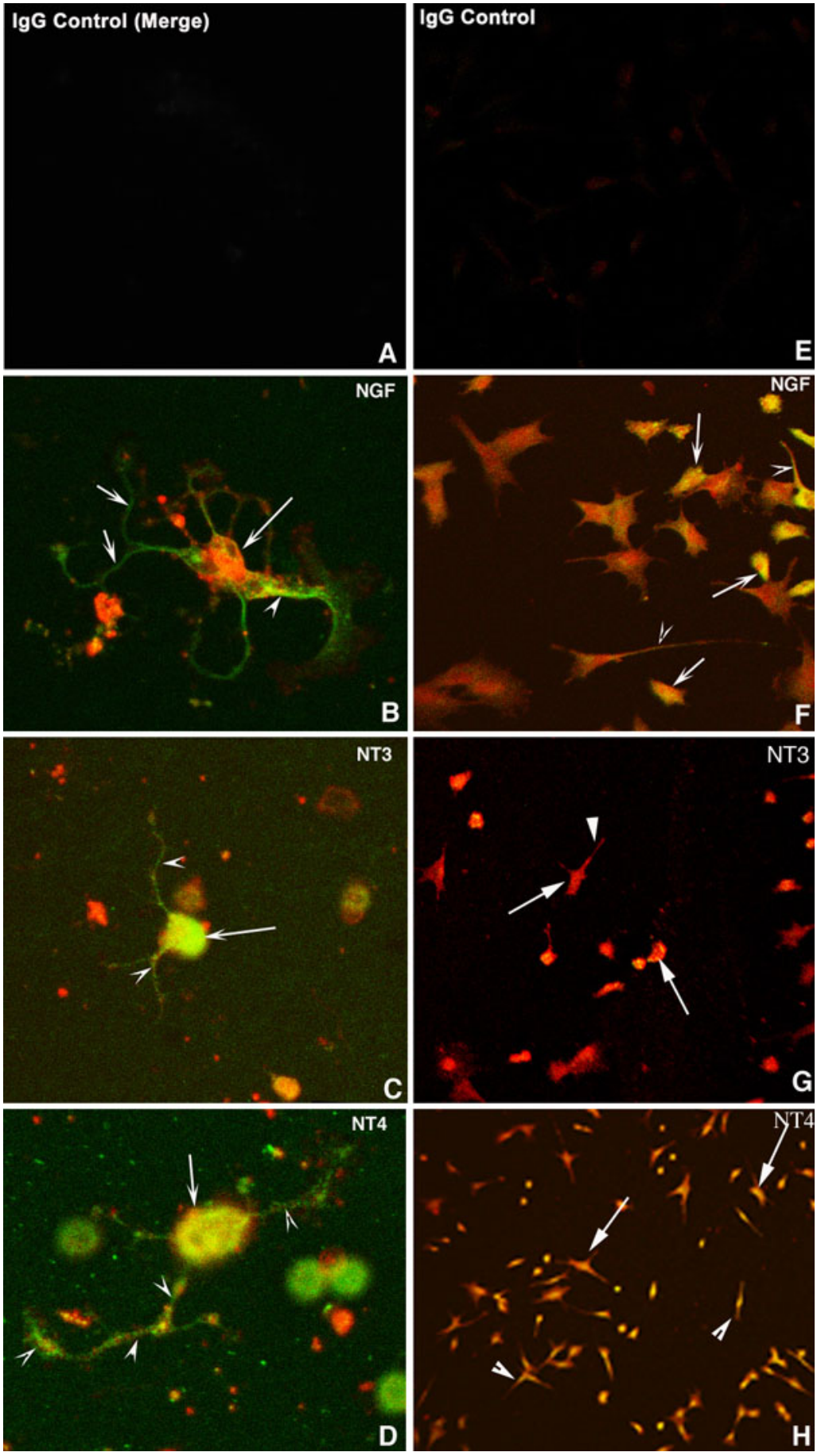

Figure 2. Confocal immunolocalization of nerve growth factor, neurotrohin-3, and neurotrophin-4. Merged images of confocal immunocytochemical localization of nerve growth factor (NGF; green), neurotrohin-3 (NT3; green), and neurotrophin-4 (NT4; green) and Thy-1 (red) in adult primary (**A**-**D**) and transformed retinal ganglion cell-5 (RGC5) cells (**F**-**H**). **B**, and **F** show the presence of NGF in the soma (arrows) and the neurites (arrowheads) of both the primary RGCs and RGC-5 cells, respectively. NGF and Thy-1 are co-expressed in the cells. Likewise, NT3 (**C** and **G**) and NT4 (**D** and **H**) are co-expressed with Thy-1 in the soma (arrows) and neurites (arrowheads) of both cell types. The IgG controls are shown for RGCs (A) and transformed RGC-5 cells (**E**).

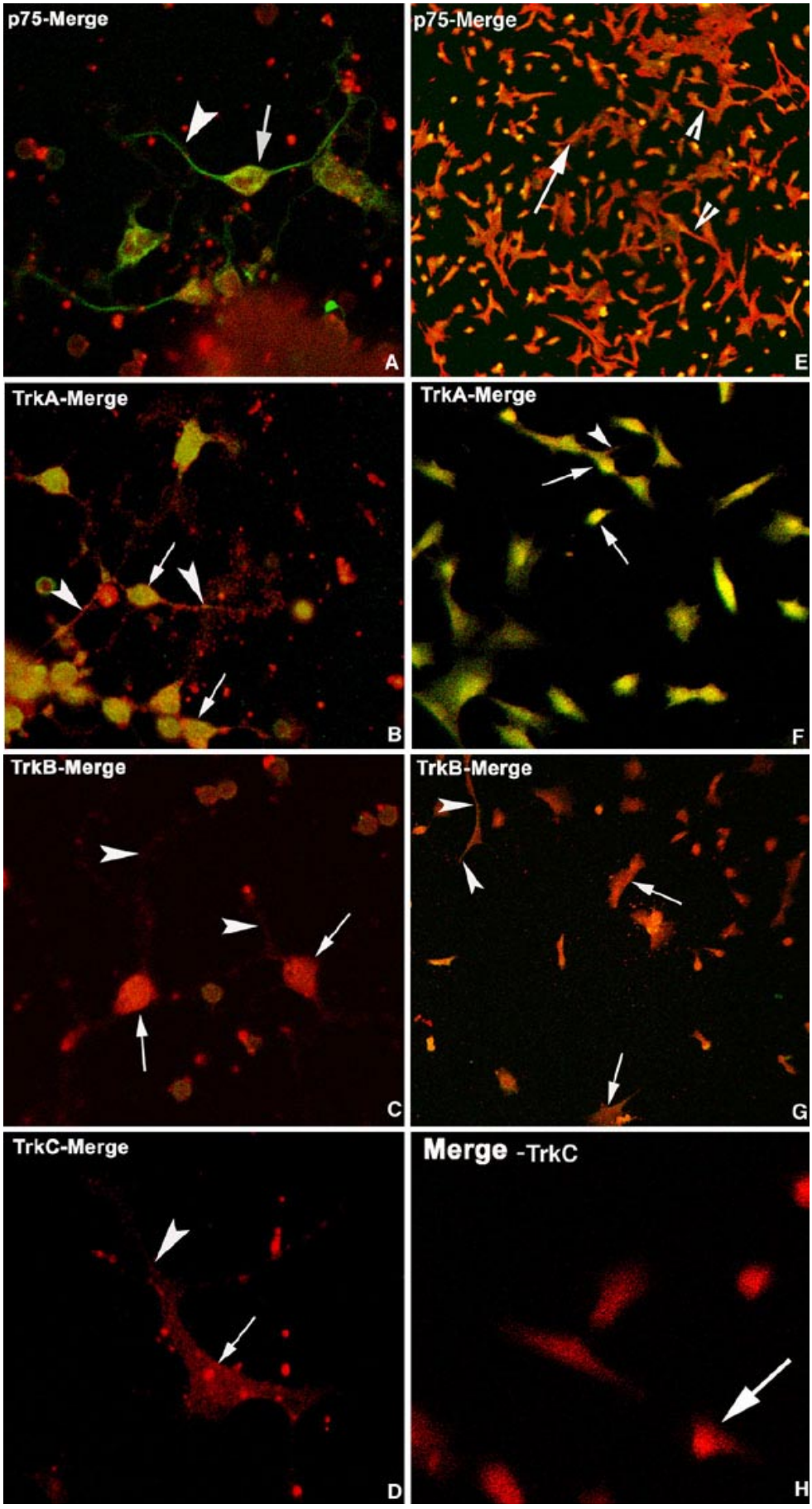

Figure 3. Confocal immunolocalization of neurotrophin receptors. Merged images of confocal immunocytochemical localization of p75 (green), TrkA (green), TrkB (green), and TrkC (green) and Thy-1 (red) in cultured adult RGCs (**A-D**, respectively) and retinal ganglion cell-5 (RGC-5) cells (**E-H**, respectively). Positive staining for p75 and TrkA with low levels of TrkB were observed in the soma (arrows) and neurites (arrowheads) of both cell types. Minimal if any, TrkC immunolabeling was detectable in either cell.

viving retinal ganglion cells at embryonic days 6 and 9 [37]. BDNF and NGF treatment protected RGCs in optic nerve crush-induced damage [17,20]. The neurotrophic factors necessary to sustain the RGCs are believed to be originated from the brain and retrogradely transported via the optic nerve to the soma of ganglion cells [10,38]. Gene therapy with BDNF was shown to be effective in neuroprotection of RGCs in a laser induced rat model of glaucoma [39].

Optic neuropathies or retinopathies that disrupt the retrograde transport of trophic factors to the RGC can cause stress to these cells and eventually their death. For example, in glaucoma, elevated intraocular pressure was shown to obstruct axonal transport at the optic nerve head, causing blockage of retrograde transport of neurotrophins [8-10,40,41]. The deprivation of these trophic factors is associated with the irreversible vision loss in glaucoma resulting from apoptosis of the RGC [6,17,40,42,43]. It is clear that neurotrophins play an important role in the health and pathology of the RGC.

In the present report, the expression profile of neurotrophins and their receptors in the RGC was determined. We show that the RGCs express BDNF, NGF, NT3, NT4, p75, TrkA, and TrkB, but minimal, if at all, TrkC. These findings in general agree with previous published reports. Using RT-PCR analysis, RGC-5 cells and rat retinas were demonstrated to express neurotrophins [14,25,26]. NGF receptor (TrkA) protein and mRNA were detected in RGCs of developing and adult rat retinas [44]. TrkB was also shown to be expressed in rat RGCs by immunohistochemistry [45]. Interestingly, the distribution of these molecules in the rat is very different from that in the porcine retina [27]. In the pig, BDNF and NT3 immunoreactivities were prominent in the RGC, while NGF was barely detectable, and no NT4 was detected. In addition, TrkA was observed in the NFL, TrkB in RGC cell bodies. The receptor p75 was widely distributed throughout the retina, and TrkC was undetectable [27]. The difference between the two species is intriguing. However, it is interesting to note that TrkC appears to be at best absent in the RGC of both animals. This is interesting observation, which needs further assessment, such that if the TrkC receptors are missing in the axons as well as the soma? Is TrkC strictly required for NT function or the NTs can function and elicit their effects by other NTRs, such as p75?

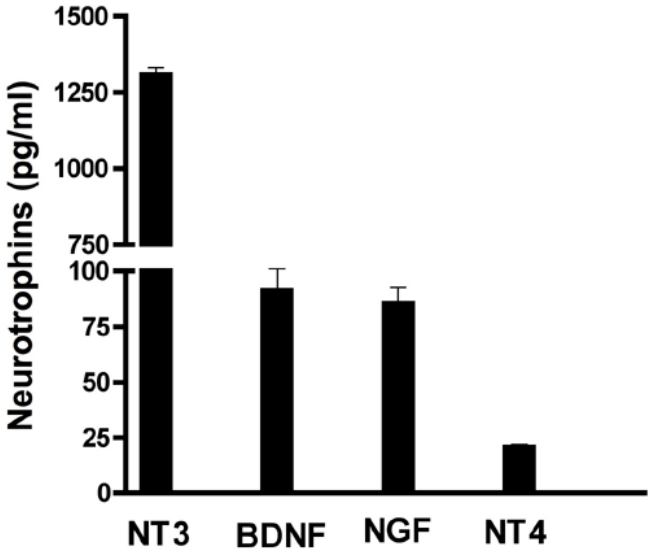

Figure 4. Secretion of neurotrophins by retinal ganglion cell-5 cells. Bar graph to show the secretion of various neurotrophins (neurotrohin-3, brain-derived neurotrophic factor, nerve growth factor, and neurotrophin-4) by retinal ganglion cell-5 cells determined by ELISA. The levels neuroptrophins were expressed as pg/ml ±SEM.

The results presented here suggest that RGCs are capable of synthesizing and secreting neurotrophins. The transformed RGC-5 cells are very similar to the cultured primary RGCs derived from adult rats in the expression pattern of neurotrophins and their receptors. Furthermore, transformed RGC-5 cells were also shown to secrete all of the neurotrophins into their culture medium. At the present time, the significance of this intrinsic production of trophic factors in the maintenance of RGC functions and survival is still unclear. Nonetheless, besides retrograde transport of neurotrophins, anterograde transport of the trophic factors has also been implicated in the survival of neurons and formation of synapses [35,46,47]. Since all four pertinent neurotrophins are expressed by RGCs, it is conceivable that they serve to ensure the normal physiology of these important cells. These results further suggest that autocrine, retrograde, and anterograde transport may all be pertinent for the maintenance of these neurons, especially in the event of a topologically separate axonal and RGC soma cell death pathways [48,49]. To the best of our knowledge, this is the first report of RGCs capability to synthesize and secrete neurotrophins in culture.

Our finding that the RGC expressed p75, TrkA, and TrkB with no or minimal expression of TrkC receptors suggests that the RGC is capable of responding to neurotrohpins produced locally in the retina. In addition to the RGC, other cells in the retina, such as Müller cells have been demonstrated to produce neurotrophins [50]. These results further support the hypothesis that both autocrine and paracrine mechanisms may be involved in the maintenance of RGCs. It will be interesting to elucidate the cellular responses to the activation of the neurotrophin receptors and their contribution to the health and disease of the RGC.

ACKNOWLEDGEMENTS

We wish to acknowledge the help of I-Fen Cheng and Dr. Shayou Chu with the confocal microscopy and Hong Zeng with the isolation of primary rat retinal ganglion cells. Financial support of American Health Assistance Foundation-Glaucoma Research Program (N.A.) and NEI, NIH (R.J.W.) is gratefully acknowledged.

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