Retrograde Neurotrophic Signaling in Rat Retinal Ganglion Cells Is Transmitted via the ERK5 but Not the ERK1/2 Pathway

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Purpose. Neurotrophic deprivation is considered an important event in glaucomatous retinal ganglion cell (RGC) death. However, the mitogen-activated protein kinase (MAPK) pathway transmitting axonal neurotrophic signals in RGC has not been identified. We investigated the involvement of ERK5 and ERK1/2 in retrograde axonal neurotrophic signaling in rats.

Methods. Adult Sprague-Dawley rats were used. Retinal immunostaining for ERK5 and MEK5 was performed. Levels of total and phosphorylated ERK5 and ERK1/2 were analyzed in retinal lysate by quantitative Western blotting. The effects of age, brain-derived neurotrophic factor (BDNF) stimulation at RGC soma (intravitreal injection) or axon ending (superior colliculus [SC] injection), axonal tyrosine kinase receptor (Trk) receptor inhibition with genistein, and acute axonal damage by optic nerve transection (ONT) were investigated at time points from 24 hours to 5 days.

Results. ERK5 and MEK5 were present in RGCs and glial cells. Phospho-ERK5 levels increased in retina and decreased in brain with age ($n = 4$; $P = 0.039$). Phosphorylation of ERK5 but not ERK1/2 was increased or decreased by SC injection of BDNF or genistein, respectively (BDNF at 48 hours [p-ERK5: $P = 0.01$; p-ERK1/2: $P = 0.55$, $n = 8$]; genistein at 48 hours [p-ERK5: $P = 0.01$; p-ERK1/2: $P = 0.5$, $n = 5$]). ONT showed a similar trend. BDNF stimulation at the RGC soma increased both p-ERK5 and p-ERK1/2 ($P = 0.035$ and $P = 0.032$, respectively; $n = 6$; at 48 hours).

Conclusions. ERK5 is present in RGCs. Retina and brain p-ERK5 levels develop differently with age. The response of ERK5 but not ERK1/2 to BDNF stimulation or inhibition at the RGC axon ending indicates that retrograde neurotrophic signals in the rat optic nerve may be mediated by the ERK5 pathway.

Keywords: extracellular signal-regulated kinase, mitogen-activated protein kinase, optic nerve, rat, retinal ganglion cell

Neurotrophins are a family of proteins secreted by neurons and glia cells. They promote survival of neighboring neurons by activation of prosurvival and inhibition of apoptotic pathways.[1] On the target cells, neurotrophins bind to tyrosine kinase (Trk) receptors, thus inducing the formation of "signaling endosomes," which are actively transported toward the nucleus.[2,3] Signaling endosomes in turn activate cascades of mitogen-activated protein kinases (MAPK),[4–6] which transfer the signal from the cytoplasm to the nucleus and activate prosurvival proteins, such as cyclic AMP-responsive element-binding protein (CREB) and myocyte-specific enhancer factor 2 (MEF2).[6] The best-characterized MAPK in neurons is extracellular signal-regulated kinases 1 and 2 (ERK1/2), which has been shown to play a major role in neuronal survival.[7–9] Recently, another MAPK, ERK5, has emerged,[10] which regulates neuronal differentiation[11–13] and the expression of neurotrophins such as brain-derived neurotrophic factor (BDNF).[14] Furthermore, ERK5 is involved in the transmission of the neurotrophic survival signal via the axon in embryonic sensory neurons.[15,16] Its downstream targets are both overlapping (CREB) and partially distinct from ERK1/2 (MEF2D and bcl-w).[15,17,18]

Neurotrophic signals derived from the target neuron and glia network of an axon play a major role in neuron selection during development.[19] Also, terminally differentiated neurons depend on the constant influx of neurotrophins to maintain their function and morphology.[20–22] However, the degree of dependence seems to vary with age, type of neuron, and neurotrophic factor.[23,24]

Deprivation of target-derived neurotrophic signals has been hypothesized to be an early pathogenic event in glaucomatous retinal ganglion cell (RGC) death.[25–29] An impairment of the axon-to-soma-directed (retrograde) axonal transport of neurotrophins has been demonstrated in various animal models of glaucoma.[30–33] However, in addition to the target-derived neurotrophins from the lateral geniculate nucleus/superior colliculus (SC; in rodents), the retina itself secrets neurotro-

phins, which directly act on the RGC soma, thus circumventing the damaged axon.[28] Moreover, in animal models of glaucoma, the retinal neurotrophin secretion and subsequently the RGC ERK1/2 pathway are stimulated.[34,35] Therefore, a qualitative difference between the axon- and soma-derived signal has been hypothesized, which allows the neuron to distinguish the locus of neurotrophic stimulation. Watson et al.[15] verified this hypothesis for rat embryonic dorsal root ganglia in vitro. In their experiments, only the MAP kinase ERK5 and not ERK1/2 was shown to transmit the axonal neurotrophic prosurvival signal, whereas stimulation at the soma activated both kinase pathways. Liu et al.[11] reported that the role of ERK5 appeared to be limited to embryonic stages in rat brains as protein levels rapidly dropped postnatally. However, Guo et al.[36] detected ERK5 in adult rat whole-retina lysate and showed an increase in protein levels in advanced glaucoma.

In this work, we sought to answer the following questions: (1) is ERK5 present in adult rat RGCs; (2) do retinal ERK5 protein levels decrease with age, as has been shown for the brain; and (3) is the axonal retrograde neurotrophic signal from the SC transmitted via the MEK5/ERK5 pathway?

Materials and Methods

Animals Used

All animals used in this study were treated in accordance with Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols were approved by the Commission on the Use of Animals in Scientific Procedures of the local government (permit number G-10/106; Tierversuchskommission, Regierungspräsidium Freiburg, Germany). Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 275 to 300 g were used for all experiments, except for the study of ERK5 in aging.

Superior Colliculus Injections

SC injections were performed as previously described.[37] Briefly, the animal was anesthetized with isoflurane and placed in a stereotactic frame. The skull was exposed, and three 0.6-mm holes were drilled 1 mm lateral to the sutura sagittalis and occipitalis with 1-mm spacing between the holes and the central hole on the level of bregma. Into each whole a volume of 0.5 μL was injected at 4.2-mm depth and, again, 0.6 μL at 4.7-mm depth, using a microliter syringe (Hamilton, Bonaduz, Switzerland). The needle was left at each position for 1 minute to allow diffusion of the substance into the tissue. All injections were administered into the right SC only. The amount injected was 3.6 ng of BDNF (carrier solution: 0.1% BSA in phosphate-buffered saline [PBS]) and 8 ng of genistein (carrier solution: 1% dimethyl sulfoxide [DMSO]/PBS). For control injections, a separate group of animals received injections of carrier solution. Experimental substance and control injections were alternated during one experimental session to avoid a systematic error.

Intravitreal Injections

The animals were anesthetized with isoflurane. The pupils were dilated with tropicamide 0.5% eye drops, and topical proxymetacaine 0.5% eye drops were administered for local anesthesia. A small piece of a microscopy coverslip was placed on the cornea with the help of lubricant eye gel (Corneregel Gel; Mann Pharma, Berlin, Germany), thus allowing direct observation of the fundus with the surgical microscope. A 3.0-μL amount of BDNF in 0.1% BSA in PBS or carrier solution was slowly injected into the vitreous approximately 1 mm posterior to the limbus with a 30-gauge needle attached to a microliter syringe (Hamilton). BDNF was injected into the left eye, control carrier solution into the right eye. During injection, the retinal perfusion was continuously observed to avoid interruption of the retinal perfusion due to fast injection. The needle was left in place before withdrawal for 1 minute to reduce reflux. Eyes with strong vitreal hemorrhage or lens damage were excluded from the study.

Optic Nerve Transection

Optic nerve transection was performed as previously described.[38] Briefly, rats were anesthetized with isoflurane. The orbit was opened through an incision at the superior orbital rim, and the optic nerve was approached by partially resecting the lacrimal gland and upper eye muscles. The optic nerve sheath was cut open longitudinally 1 to 2 mm posterior to the globe, while care was taken not to damage blood vessels. The optic nerve was cut with scissors. Before wound closure, the retinal perfusion was ascertained fundoscopically. Animals with severe reduction of perfusion were excluded.

Western Blots

All Western blots were produced twice for each sample, and the results were averaged. Proteins were separated on a 10% acrylamide SDS gel followed by wet blotting protein transfer onto a polyvinylidene fluoride membrane (Immobilon-P; GE Healthcare, Munich, Germany). The membrane was blocked in 5% BSA in TBS/Tween 0.1% (TBS-T) solution for 60 minutes at room temperature. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. Antibodies used were p-ERK5, 1:1000 dilution; total ERK5, 1:2000 dilution; p-ERK1/2, 1:2000 dilution; total ERK1/2, 1:2000 dilution; p-CREB, 1:1000 dilution (all Cell Signaling/New England Biolabs, Frankfurt, Germany); β-actin 1:20,000 dilution (Abcam, Cambridge, UK). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG diluted 1:20,000 in TBS-T (ECL; Amersham/GE Healthcare). Protein bands were visualized with chemiluminescent reaction (ECL-Prime kit; GE Healthcare) and scanned with an electronic gel documentation system (ChemoCam; Intas, Göttingen, Germany).

Quantification of band intensity was performed with an ImageJ software gel analyzer plug-in (http://rsbweb.nih.gov/ij/).

All band intensities were normalized to that of β-actin. Results are means ± SEM. Bonferroni-Holm correction for multiple comparisons was applied.

For the comparison of protein level changes with age, a bootstrap analysis was performed using R version 3.0.2 software (The R Foundation for Statistical Computing, Vienna, Austria). Western blotted protein levels of samples belonging to a certain age and tissue group were randomly selected for calculation of the ratio $R = (\text{retina old}/\text{retina young})/(\text{brain old}/\text{brian young})$. The calculation with random samples for each group was repeated 1 million times. To exclude repeat permutations, the "unique" command was applied to the data set, leaving 256 "unique" results. The number (N) of results with $R > 1$ or $R < 1$ was counted. The P value for the hypothesis that the change with age is higher in retina tissue ($R > 1$) was calculated with $P = N(R > 1)/N(R > 1, R < 1)$. A P value of <0.05 was considered statistically significant.

Immunohistochemistry

The eyes were enucleated and incised at the cornea to improve fixation while immersed in 4% paraformaldehyde solution for 4 hours. Subsequent incubation in 30% sucrose solution for 4

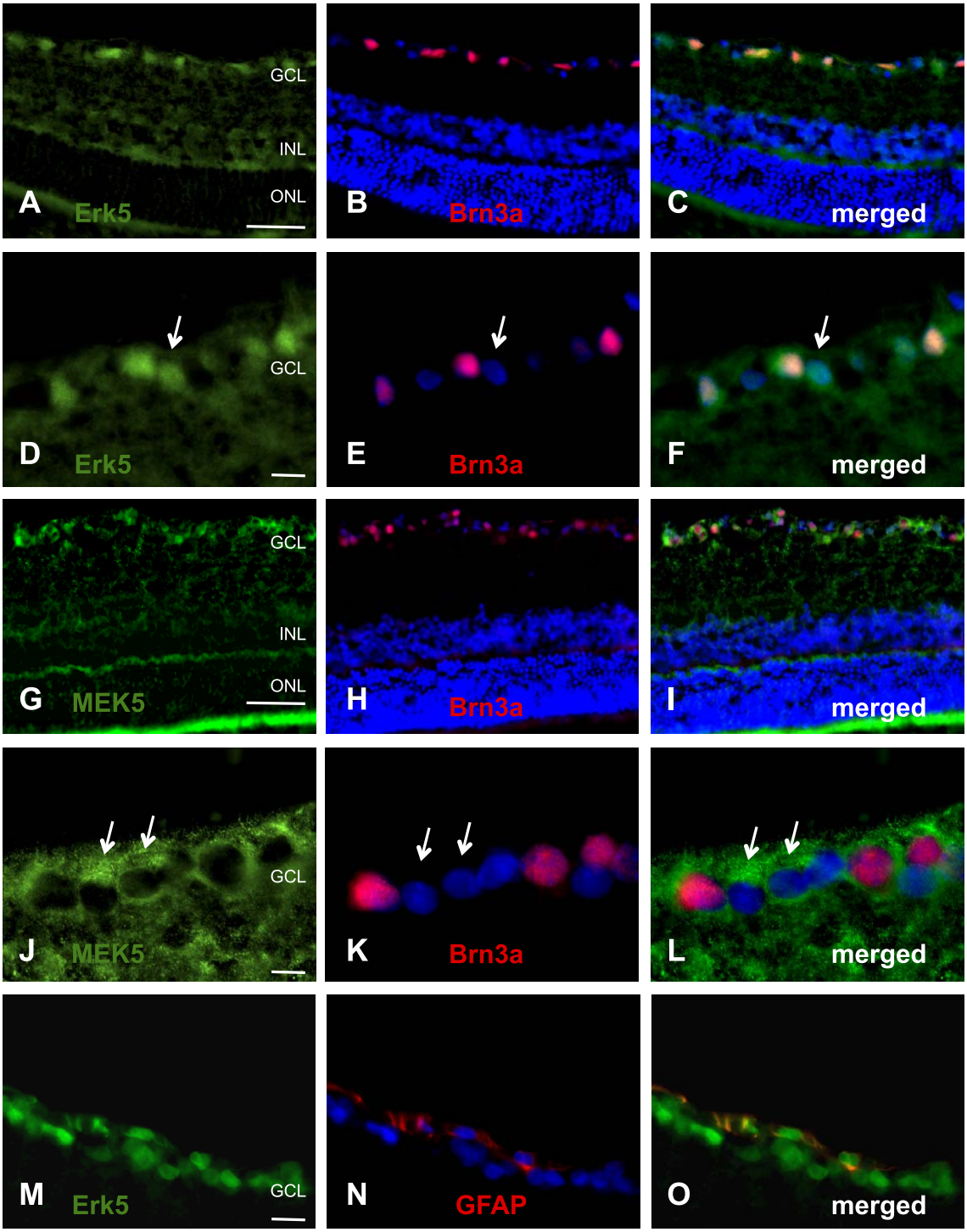

Figure 1. Localization of ERK5 is shown in the adult rat retina. In each image, the ganglion cell layer is facing up. The ERK5- and MEK5 signal is always green (*left column*), red signals are from the RGC marker Brn3a (*center column* in [**B**, **E**, **H**, **K**]) or GFAP (**N**). DAPI-stained nuclei are blue (*center* and *right columns*). The *right column* shows the merged images of each row. (**A–F**) ERK5 staining is shown in an overview ([**A–C**]; magnification: ×200; *scale bar*: 50 μm) and GCL ([**D–F**]; magnification: ×630; *scale bar*: 10 μm). The strongest signal is present in the GCL, consisting of Brn3a-positive RGC and Brn3a-negative cells of the same shape, presumably displaced amacrine cells (*white arrows* in [**D–F**]). In addition, cell bodies in the inner and outer nuclear layers were stained. (**G–L**) MEK5 staining is shown in an overview ([**G–I**]; magnification: ×200) and GCL ([**J–L**]; magnification: ×630). Corresponding to ERK5, cells of the GCL showed the strongest staining in the retina. As with ERK5, these cells are either Brn3a-positive RGC or Brn3a-negative cells of the same localization and shape (*white arrows* in [**J–L**]), which are considered displaced amacrine cells. The intracellular distribution is different, with a purely cytoplasmic localization of MEK5. (**M–O**) ERK5 is present in GFAP-positive astroglia of the inner retina (magnification: ×630).

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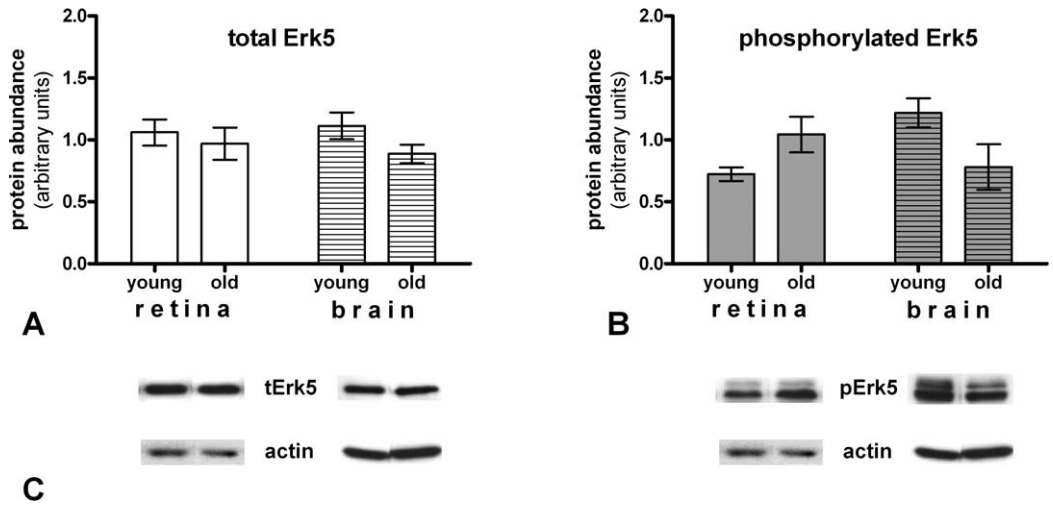

FIGURE 2. Levels of total (**A**) and phosphorylated (**B**) ERK5 are shown in retina and brain tissues of young (3-week-old) and elderly (>12-month-old) rats. The protein levels were normalized with the β-actin levels of the same Western blot lane. Differences between the age-related changes of p-ERK5 protein levels in retina and those in brain tissue were statistically significant as determined by bootstrap analysis for p-ERK5 ($P = 0.039$). (**C**) Representative Western blots are shown for each tissue and age group. Blots are aligned with the corresponding bars of the plots (**A**, **B**). The total- and p-ERK5 blots are from the same two animals and the same blotting membrane, thus, the actin bands are the same for both isoforms.

hours was added for tissue cryoprotection. The eyes were then embedded in OCT medium and cryosectioned to 10-μm thickness.

For immunostaining, the sections were blocked with 5% BSA and 0.3% Triton X-100 in PBS solution at room temperature for 10 minutes. The primary antibody was applied in blocking buffer, the secondary antibody (Alexa-488 or -568 conjugated; Invitrogen/Life Technologies, Darmstadt, Germany) diluted 1:1000 in PBS. Primary antibodies were: ERK5, 1:100 dilution (Santa Cruz Biotechnology, Heidelberg, Germany), MEK5, 1:200 dilution (Cell Signaling), glial fibrillary acidic protein (GFAP), 1:500 dilution (Neomarkers; Thermo Fisher Scientific, Schwerte, Germany), vimentin, 1:500 dilution (Sigma-Aldrich, Munich, Germany); Brn3a, 1:100 dilution (Santa Cruz Biotechnology). Images were processed with ImageJ software. Apart from a background subtraction routine (rolling ball radius of 200 pixels in 1384- × 1040-pixel images), no nonlinear adjustments were made.

RESULTS

Distribution of ERK5 and MEK5 in Adult Rat Retina

Immunostaining for ERK5 and MEK5, the kinase by which ERK5 is exclusively phosphorylated, confirmed the presence of both proteins in the retina of adult rats (Figs. 1A–L). The strongest staining was located in the ganglion cell layer (GLC). Colabeling with Brn3a, an RGC marker, confirmed that all RGC were ERK5/MEK5-positive. In addition, there was a subset of morphologically identical ERK5/MEK5-positive cells, which were Brn3a-negative. These cells were considered displaced amacrine cells (Fig. 1, white arrows). Weaker staining was observed in the cells of the inner and outer nuclear layers. Corresponding to the sequence of activation and the transport direction of the neurotrophic signal, the intracellular distribution differed between ERK5 and MEK5. The latter appeared only in the cytoplasm (Figs. 1J–1L), whereas ERK5 was detected in both the cytoplasm and the nucleus, with a tendency toward stronger nuclear signals (Figs. 1D–F). The staining showed a granular pattern, particularly for MEK5.

In addition to being detected in neuronal cells, ERK5 was detected in astroglia of the inner retina as identified by colabeling with (GFAP; Figs. 1M–O). By colabeling with vimentin, no clearly distinguishable signal could be detected from Müller glia (data not shown). However, due to the high density of cells in the inner nuclear layer, a weak staining might have been missed, had we not been using a confocal microscope.

Age-Dependent Changes in ERK5 Protein Levels in Retina and Brain

Given previous observations showing a rapid decline of ERK5 levels in rat brain after birth,[11] we investigated whether a similar trend existed later in life in both the retina and the brain. Samples of whole retina and brain parietal cortex lysate of young (3-week-old) and elderly (12- to 15-month-old) rats ($n = 4$ for each group) were compared with respect to the protein levels of total and p-ERK5. Age-dependent change was statistically analyzed by using bootstrap analysis, described in Materials and Methods. In both tissues the level of total ERK5 did not significantly change with age (Figs. 2A, 2C). However, when p-ERK5 was analyzed, retinal levels were found to be increased and brain levels were decreased with age (Figs. 2B, 2C). The differences between the age-dependent changes were statistically significant ($P = 0.039$).

Transmission of Retrograde Neurotrophic Signals via the MEK5/ERK5 Pathway

We investigated the involvement of the MEK5/ERK5 pathway in retrograde neurotrophic signaling to the RGC soma by injection of 3.6 ng of BDNF (or carrier solution in control animals) unilaterally into the SC of adult rats. Subsequently, levels of activated (i.e., phosphorylated) ERK5 and ERK1/2 in the retina were determined by quantitative Western blotting from whole-retina lysate. Protein levels were normalized to that of β-actin and expressed as relative quantity (RQ), which is the BDNF stimulated tissue-to-control tissue ratio. Thus, an RQ of >1 indicated an increase in the respective proteins after BDNF injection and an RQ of <1 a decrease in the protein levels. Levels of total and phosphorylated ERK5 and ERK1/2 were analyzed at 24 and 48 hours after injection (Figs. 3A–C; $n = 8$ for the BDNF and control group at each time point).

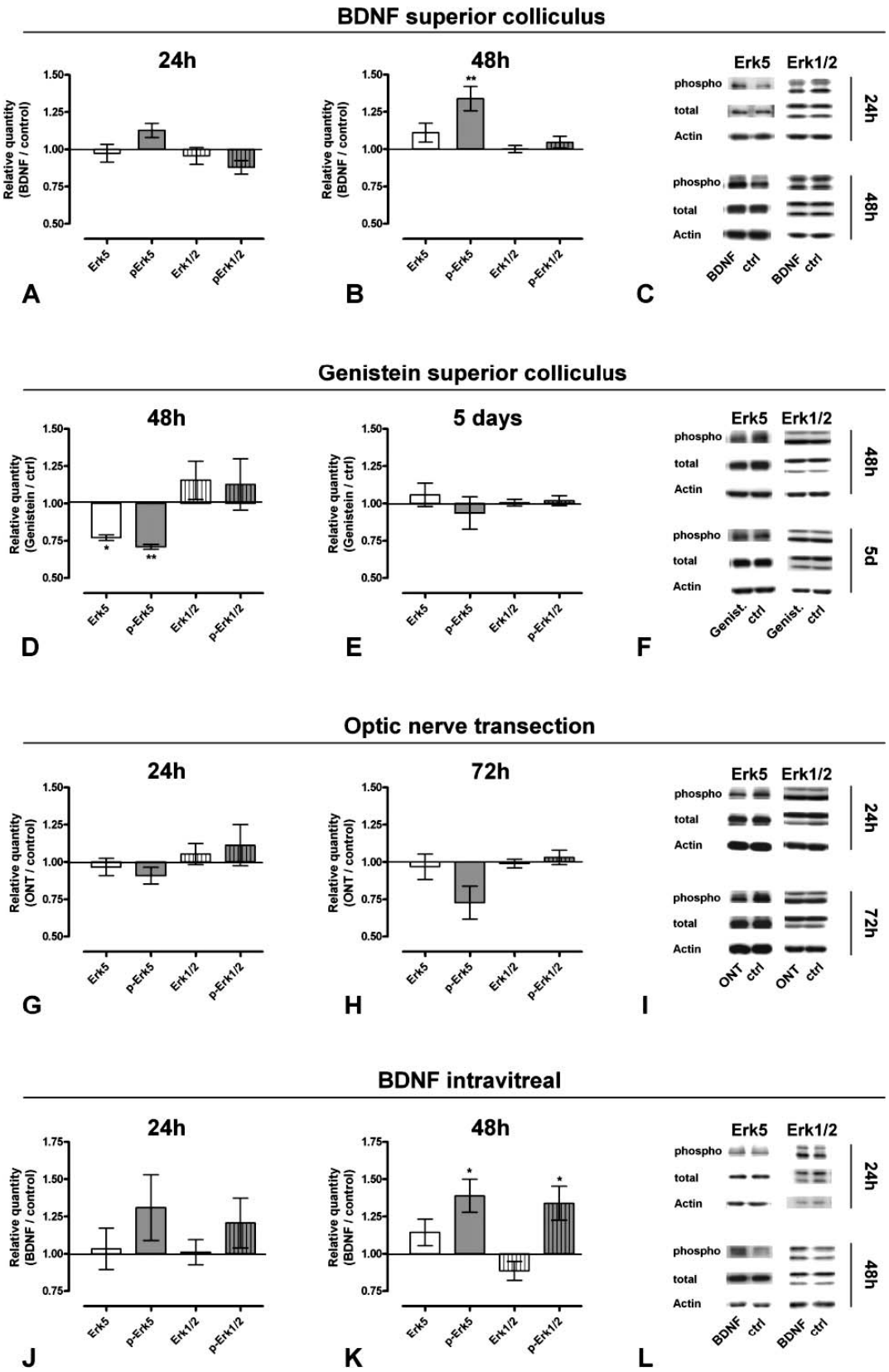

Figure 3. Retinal protein levels of ERK5 and ERK1/2 and its phospho isoforms as measured with quantitative Western blotting are shown after different experimental procedures were performed to target the neurotrophic signaling of either the RGC axon (*top three rows*) or the RGC soma (*bottom row*). *Bar graphs* show the relative quantity (RQ = ratio of experimental and control eye) of the β-actin normalized protein levels. An RQ of >1 indicates the experimental procedure increased the protein level; an RQ of <1 signifies a decrease. The *right column* shows representative Western blots of each row. (**A–C**) Neurotrophic stimulation with BDNF at the RGC axon ending in the SC led to a significant increase in ERK5 but not ERK1/2 phosphorylation in the retina 48 hours after injection ($P = 0.01$; $n = 8$). (**D–F**) Inhibition of endogenous neurotrophic signaling at the

axon ending with genistein significantly reduced phosphorylated and total ERK5 but not p-ERK1/2 levels in the retina 48 hours after injection ($P = 0.01$ and $P = 0.02$, respectively; $n = 5$). At 5 days, both phosphorylated and total ERK5 had returned to control levels ($P = 0.99$ for both protein isoforms; $n = 4$). **(G–I)** Complete interruption of the retrograde neurotrophic signal by optic nerve transection also appeared to lower p-ERK5 levels in the retina, with relatively smaller change in p-ERK1/2 levels. However, due to high variability, the results were not statistically significant (72h time point; $P = 0.27$ and $P = 0.99$ for p-ERK5 and p-ERK1/2, respectively; $n = 5$). **(J–L)** Neurotrophic stimulation of RGC somata in the retina by intravitreal injection of BDNF significantly increased both p-ERK5 and p-ERK1/2 levels at the 48 hours time point ($P = 0.035$ and $P = 0.032$, respectively; $n = 6$).

At both time points, ERK1/2 was not activated (RQ of p-ERK1/2 at 24 hours: 0.88 ± 0.045, $P = 0.09$; RQ at 48 hours: 1.05 ± 0.04, $P = 0.52$). However, p-ERK5 showed a trend toward higher levels at the 24-hour time point (RQ of 1.13 ± 0.047, $P = 0.09$; Fig. 3A) and was significantly increased at 48 hours after BDNF injection (RQ of 1.34 ± 0.082, $P = 0.01$; Fig. 3B). For both ERK1/2 and ERK5, the levels of the total protein did not change at either time point (ERK1/2 at 24 hours: 0.96 ± 0.057, $P = 0.9$; ERK1/2 at 48 hours: 1.0 ± 0.024, $P = 0.99$; ERK5 at 24 hours: 0.97 ± 0.06, $P = 0.67$; ERK5 at 48 hours: 1.11 ± 0.063, $P = 0.33$; Figs. 3A, 3B).

To test whether inhibition of the endogenous neurotrophic support at the RGC axon ending would lead to inverse results as with BDNF stimulation, genistein was injected into the SC, and retinal lysate was analyzed after 48 hours ($n = 5$; Figs. 3D–F). Genistein is a Trk inhibitor. Its inhibitory effect includes Trk receptor phosphorylation, which is one of the initial steps in neurotrophic signaling, leading to both formation of the signaling endosome and activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway[1,2] (if present).

Genistein applied to the RGC distal axon led to a decrease in retinal p-ERK5 levels at the 48-hour time point (RQ of 0.71 ± 0.016, $P = 0.01$; Fig. 3D). Furthermore, the level of total ERK5 dropped significantly (RQ of 0.77 ± 0.018, $P = 0.02$). Similar to the BDNF stimulation experiment, p-ERK1/2 and total ERK1/2 levels did not change significantly (RQ of p-ERK1/2: 1.13 ± 0.17, $P = 0.54$; RQ of total ERK1/2: 1.15 ± 0.128, $P = 0.7$; Fig. 3D). To test, whether the decrease in total ERK5 protein persisted throughout the following days, the experiment was repeated with a 5-day time point ($n = 4$; Fig. 3E). At that point, both total and p-ERK5 had returned to levels similar to those in control animals (RQ of total ERK5: 1.06 ± 0.08, $P = 0.99$; RQ of p-ERK5: 0.94 ± 0.11, $P = 0.99$).

In order to completely interrupt axonal ERK5 activation in RGC, optic nerve transection was performed, and retinal lysate was analyzed at 24 ($n = 4$) and at 72 hours ($n = 5$) postlesion (Figs. 3G–I). At both time points, the RQ of p-ERK5 levels showed a trend toward lower values, and the mean decrease in p-ERK5 levels was greater at the 72- than at the 24-hour time point (24 hours: 0.91 ± 0.056, $P = 0.8$ [Fig. 3G]; and at 72 hours: 0.73 ± 0.11, $P = 0.27$ [Fig. 3H]). However, due to the relatively high variability of the results, the changes were not statistically significant. Analogous to the previous results, the amplitude of change of p-ERK5 appeared higher than the change of p-ERK1/2 levels (RQ of p-ERK1/2 at 24 hours: 1.11 ± 0.14, $P = 0.99$ [Fig. 3G]; and at 72 hours: 1.03 ± 0.048, $P = 0.99$ [Fig. 3H]).

Next, we investigated whether the application of BDNF to the RGC soma and proximal axon led to a different pattern of MAPK activation (Figs. 3J–L). The levels of total and phosphorylated ERK5 and ERK1/2 were determined at 24 hours (Fig. 3J; $n = 7$) and at 48 hours (Fig. 3K; $n = 6$) after intravitreal injection of BDNF or carrier solution. In contrast to the application of BDNF to the distal axon (Fig. 3B), now both ERK5 and ERK1/2 were activated. The elevation relative to control was statistically significant for both phosphorylated proteins at the 48-hour time point (RQ of p-ERK5 at 24 hours: 1.31 ± 0.22, $P = 0.84$; and of p-ERK5 at 48 hours: 1.39 ± 0.11, $P = 0.035$; and of p-ERK1/2 at 24 hours: 1.21 ± 0.16, $P = 0.78$; and of p-ERK1/2 at 48 hours: 1.34 ± 0.11, $P = 0.032$). The levels of total ERK5 and ERK1/2 did not change significantly (Figs. 3J, 3K; RQ of total ERK5 at 24 hours: 1.03 ± 0.14, $P = 0.9$; and of total ERK5 at 48 hours: 1.14 ± 0.09, $P = 0.17$; and of total ERK1/2 at 24 hours: 1.01 ± 0.08, $P = 0.91$; and of total ERK1/2 at 48 hours: 0.88 ± 0.06, $P = 0.24$).

Discussion

In this work we have demonstrated that ERK5 is present in adult rat RGCs and that, compared to the level in brain, the level of p-ERK5 increases with age. Furthermore, the level of activation of the MEK5/ERK5 pathway is related to the neurotrophic input at the RGC axon ending in the SC. The classic ERK1/2 MAP kinase pathway, which, more than ERK5, has been an object of research of neurotrophic support and neuroprotection in RGCs,[7–9] was not involved in axonal neurotrophic signaling. However, both the ERK 5 and ERK1/2 pathways were activated upon neurotrophic stimulation at the RGC soma and proximal axon. These findings are in accord with previous in vitro experiments with embryonic rat dorsal root ganglia.[15]

In addition to demonstrating that ERK5 activation is related to neurotrophic stimulation at the axon ending, we cannot currently provide data for the relevance of this axonal ERK5 pathway for RGC survival. A targeted inactivation of the axon-derived ERK5 activation would be desirable to answer this question. However, the fact that ERK5 can be activated by both soma and axon-derived neurotrophic signaling makes this goal very difficult to achieve; a knockdown of the whole cellular ERK5 expression would be unspecific for the axonal pathway. In our hands, the attempt to specifically inhibit axonal MEK5 activation by injection of a MEK5-specific inhibitor (BIX02188; Boehringer-Ingelheim, Ridgefield, CT) into the SC had no significant effect on retinal p-ERK5 and p-CREB levels (2.4 μg of BIX02188 in 10% DMSO/PBS or carrier solution in control animals) injected into the SC. Analysis of whole-retina lysate 24 hours after injection ($n = 10$ for BIX02188 and control group; RQ of p-ERK5: 0.98 ± 0.076, $P = 0.79$; RQ of p-CREB: 1.26 ± 0.22, $P = 0.54$). Among the various reasons for this approach to fail were the relatively fast decay of the BIX02188 inhibitory effect in vivo,[39] the need to use 10% DMSO as solvent, and the conceivable failure of the inhibitor to reach MEK5 kinases further proximal in the axon, might have played a major role. Although the failure of our inhibitor-based approach made it impossible for us to proof in vivo the relevance of axonal ERK5 activation for RGC survival, in vitro data from Watson et al.,[15] using rat embryonic dorsal root ganglia, strongly suggested that MEK5/ERK5 dysfunction has implications for cell survival.

A general problem, which applies to all our experiments, is the fact that RGC represent only a small cell population in the retina, which leads to an unfavorable signal-to-noise ratio when analyzing whole-retina lysate, as was done in this study. This could partially explain the small amplitude of change of the p-ERK5 levels following stimulation or inhibition of the neurotrophic signaling at the RGC axon ending. The use of immunohistochemistry with quantification of the signal intensity might have allowed us to detect protein changes specifically in RGCs. However, despite the general problems of quantitative immunohistochemistry, the ERK5 phosphoryla-

tion motif (TEY) is identical to that of ERK1/2, thus leading to cross-reactivity of p-ERK5 antibodies with ERK1/2 protein in immunostaining. We therefore decided to use only quantitative Western blotting, which allows specific identification of the p-ERK5 signal.

Interestingly, the drop in p-ERK5 levels after optic nerve transection (ONT) did not exceed the approximately 25% up or down amplitude of the other, less invasive pharmacological experiments at the RGC axon. The two facts discussed above may partially explain this phenomenon: (1) the ERK5 activation pathway via the RGC soma and (2) the presence of other cell populations expressing ERK5. In addition, the particularly high variability of the phospho-ERK5 levels might have been caused by occasional intraoperative alterations of the retinal blood supply due to short time stretching of the optic nerve and its blood vessels during dissection, which in turn could have activated MAPK. An unspecific drop in ERK5 protein levels due to RGC death after ONT is a very unlikely scenario in our experiments, given the relatively short observation time points (1 and 3 days). Several studies have indicated that significant cell loss does not occur before day 4.[40–42] Furthermore, at day 3 only p-ERK5 is reduced but total ERK5 levels are unchanged, suggesting a change in phosphorylation state but not in total amount of the protein.

The Trk inhibitor genistein, used for inhibition of neurotrophic signaling at the axon ending, probably affected various signaling pathways in SC neurons and glia cells. Thus, alterations of neurotrophin secretion in the SC might have occurred, which in turn might have indirectly and additionally influenced Erk5 levels in the retina.

The question of relevance of the MEK5/ERK5 pathway is closely related to the question of whether other pathways of neurotrophic signaling exist along the axon. We have investigated the role of ERK MAP kinases after stimulation with BDNF, which is considered a major neurotrophic pathway in RGCs. However, other neurotrophic factors, such as NGF and NT-4/5, are present in the SC.[43] Whether their signals also converge to the ERK5 MAP kinase remains to be investigated.

Besides MAP kinase pathways, neurotrophic survival signals can also be transmitted via the PI3K/AKT cascade.[1] This is true not only for neurotrophic stimulation at the cell body but also has been described for neurotrophins selectively applied to the distal axon of primary cultured rat sympathetic neurons.[44,45] Thus, PI3K/AKT may, in addition to ERK5, mediate retrograde survival signals in rat RGCs. Investigating this pathway was outside the scope of this project but is certainly an important issue to be addressed in future research.

The hypothesis that RGCs are potentially able to detect the locus of neurotrophic support through differential activation of MAP kinases is supported by our finding that the activation of ERK5 and ERK1/2 depend on the site of BDNF application. However, the fact that the ERK5 pathway can be activated by stimulation at both the soma and the distal axon makes it puzzling to hypothetically name the exact parameter that provides the neuron information about the level of neurotrophic stimulation at either site. Given our previous results and those from previous work,[15,16] we hypothesize that the ERK5-to-ERK1/2 ratio may reflect the balance of neurotrophic stimulation between cell body and distal axon.

Considering our results in light of the hypothesis of retrograde axonal neurotrophic deprivation as an early event in glaucoma pathogenesis, the hypothesis may be specified, now saying that, as a consequence of glaucomatous axon damage, an impairment of ERK5 activation via the axon may occur, which leads to a decrease in prosurvival signals that, in concert with other factors, promotes RGC apoptosis. Consequently, future research of the role of neurotrophic deprivation in glaucoma models as well as neuroprotection studies should take into consideration the level of ERK5 activation in RGCs.

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