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Changes in fibroblast growth factor-2 and FGF receptors in the frog visual system during optic nerve regeneration

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Abstract

We have previously shown that application of fibroblast growth factor-2 (FGF-2) to cut optic nerve axons enhances retinal ganglion cell (RGC) survival in the adult frog visual system. These actions are mediated via activation of its high affinity receptor FGFR1, enhanced BDNF and TrkB expression, increased CREB phosphorylation, and by promoting MAPK and PKA signaling pathways. The role of endogenous FGF-2 in this system is less well understood. In this study, we determine the distribution of FGF-2 and its receptors in normal animals and in animals at different times after optic nerve cut. Immunohistochemistry and Western blot analysis were conducted using specific antibodies against FGF-2 and its receptors in control retinas and optic tecta, and after one, three, and six weeks post nerve injury. FGF-2 was transiently increased in the retina while it was reduced in the optic tectum just one week after optic nerve transection. Axotomy induced a prolonged upregulation of FGFR1 and FGFR3 in both retina and tectum. FGFR4 levels decreased in the retina shortly after axotomy, whereas a significant increase was detected in the optic tectum. FGFR2 distribution was not affected by the optic nerve lesion. Changes in the presence of these proteins after axotomy suggest a potential role during regeneration.

Keywords

FGF-2; CNS; axotomy; FGFR; optic nerve; retina; RGC; regeneration; growth factor; axon

Introduction

Within the visual system, fibroblast growth factor-2 (FGF-2) has been identified as a crucial molecule for the regulation of important retinal processes. FGF-2 stimulates regeneration in embryonic retina by transdifferentiation of retinal pigmented epithelium (Tsonis and Del Rio-Tsonis, 2004; Spence et al., 2007; Vergara and Del Rio-Tsonis, 2009) and via activation of stem/progenitor cells in the ciliary body and ciliary marginal zone (Spence et al., 2004). It has been reported that FGF-2 mediates protection against oxidative stress in retinas, probably by decelerating the rate of photoreceptor death (Yu et al., 2004). FGF-2's effect on photoreceptor survival has also been demonstrated in vitro (Fontaine et al., 1998; Kinkl et al., 2001; Traverso et al., 2003).

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FGF-2 also stimulates retinal ganglion cell (RGC) differentiation and confers protection from developmental cell death (Desire et al., 1998), and has survival-promoting effects on RGCs in vitro (Bahr et al., 1989) and in vivo after axotomy (Sievers et al., 1987). FGF-2 also stimulates and directs axonal growth of developing retinal ganglion cells (Brittis et al., 1996; McFarlane et al., 1995, 1996; Webber et al., 2003), and the regrowth of injured RGC axons in the adult retina (Sapieha et al., 2003).

Fibroblast growth factor receptors (FGFRs) are normally present in retinal neurons of the mouse (Catalani et al., 2008), rat (Blanquet et al., 1996; Kinkl et al., 2002), primate (Cornish et al., 2004), chicken (Heuer et al., 1990; Ohuchi et al., 1994; Tcheng et al., 1994), cow (Torrìglia et al., 1994), zebrafish (Hochmann et al., 2012), *Xenopus* (McFarlane et al., 1995; Vergara and Del Rio-Tsonis, 2009;) and human (Fuhrmann et al., 1999). Increased levels of both FGF-2 and its receptors have been reported in response to injury or disease in the visual system (Kostyk et al., 1994; Gao and Hollyfield, 1995; Wen et al., 1995; Cao et al., 1997; Yamamoto et al., 1996; Guillonneau et al., 1998; Cao et al., 2001; Casson et al., 2004; Yu et al., 2004; Valter et al., 2005; Lonngren et al., 2006).

We have previously shown that FGF-2 treatment to cut optic nerve axons of adult *Rana pipiens* significantly improves RGC survival rate after axotomy (Blanco et al., 2000). Much of this effect appears to be through FGFR1 activation and upregulation of retinal brain-derived neurotrophic factor (BDNF) expression, while enhancing activation of MAPK and PKA intracellular pathways at early stages after axotomy (Rios-Muñoz et al., 2005, Soto et al., 2006b).

Although we have a detailed picture of how exogenously-applied FGF-2 enhances RGC survival after injury in the visual system of the frog, the role of endogenous FGF-2 in this system is less well understood. In this study we determine the distribution of the growth factor and its receptors in the retina and optic tectum before, and after, inducing a lesion to the optic nerve. Our results show that FGF-2 and receptors are normally present in subpopulations of cells in the retina and in cells of the optic tectum, and that axotomy increases the amounts of the factor and its receptors in both regions during the period in which regeneration is occurring. These findings are consistent with a potential role of endogenous FGF-2 signaling in the regenerative process that naturally occurs in the amphibian visual system after injury.

Materials and methods

Animals

Adult frogs (*Rana pipiens*) of both sexes were used. They were obtained from commercial sources and kept in tanks with recirculating tap water at 18°C.

Optic nerve lesion

Under tricaine anesthesia, the right eyeball of a series of frogs was approached from the palate and an incision was made. The extraocular muscles were teased apart and the intraorbital section of the optic nerve was exposed, and then cut. The incision was sutured and the animals allowed to recover for several hours in the laboratory under observation before replacing them in their tanks in the animal facility. All our protocols have been approved by IACUC and follow the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and comply with EU Directive 2010/63/EU for animal experiments.

Immunohistochemistry

Several eyes and tecta of control and experimental frogs of 1, 3 and 6 weeks after inducing the lesion in the optic nerve, were fixed by transcardial perfusion with 2% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). After dissection, some tissues were fixed in the paraformaldehyde solution for 15 minutes (FGF-2), and others for 1 hour (FGFR1-4), washed in buffer (PBS 0.1 M), and placed in 30% sucrose for cryoprotection at 4° C overnight. Cryostat sections were incubated with polyclonal antibodies against FGF-2 (1:200, Oncogene), glutamine synthetase (Chemicon International Inc., 1:500), FGFR1 (1:100), FGFR3 (1:200), FGFR4 (1:300) (Santa Cruz Biotechnology) and FGFR2 (1:500, Sigma) overnight at 4° C. After washing, sections were incubated for two hours in a goat anti-rabbit secondary antibody coupled to CY3 (Jackson Immunoresearch) and mounted in Polymount. Antibody specificity was tested by omitting the primary antibody and by preabsorbing with the corresponding specific peptides. These procedures resulted in the absence of immunostaining. In order to try to ensure consistent antibody staining, frozen sections from control animals, and from animals at different experimental times were processed together, using the same antibody dilutions. Imaging settings were the same for each series.

TDA Retrograde Labeling of RGCs

The retrograde labeling of RGCs with tetramethylrhodamine dextran amine (TDA) was carried out as follows. A 1mm square piece of cellulose acetate membrane filter (0.8 µm pore size, Schleicher and Schuell, Keene, NH) was infused with a saturated solution of tetramethylrhodamine dextran amine (TDA, 3000 MW, Molecular Probes) in ethanol and left to air-dry so that particles of TDA were embedded within the filter. The optic nerves were exposed and cut close to the back of the eyeball. The piece of filter was inserted into the stump and the palate was sutured. After approximately 48 hours, the labeled retinas were dissected and fixed as described above, and processed for immunohistochemistry. With these parameters, TDA consistently labeled 80–84% of the cells in the GCL of control retinas. The percentage of displaced amacrine cells in *Rana pipiens* retina has been previously calculated as 16% (Scalia et al. 1985), so we appear to be labeling most of the RGCs.

Total protein isolation from retinal and tectal tissue

A total of four pools of each control and experimental (1 week, 3 weeks, and 6 weeks after axotomy) tissue was produced from two animals each per pool. Isolated tissue was homogenized in lysis buffer containing 10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1/100 per volume of protease inhibitor cocktail (0.1 µg/mL leupeptin, 0.001 µg/mL pepstatin, 0.1 µg/mL aprotinin), and 1/100 per volume of phosphatase inhibitor cocktail I and II (Sigma) using a motorized homogenizer. Cells were disrupted by sonication for 10 s (1 pulse per s at maximum power) using a Sonic Dismembrator (Fisher Scientific) at 4°C. Samples were then left to stand for 30 min at 4°C. Protein concentration was determined using a Lowry-based assay from Bio-Rad (DC-protein assay; Bio-Rad).

Western blotting

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Approximately 50 µg of total protein from each sample was separated in a 4–20% gel (Bio-Rad). Electrophoresed proteins were then transferred to a polyvinylidene difluoride membrane (Millipore) and blocked for 2 h. Membranes were then incubated overnight at 4°C with the following rabbit polyclonal antibodies: anti-FGF-2, anti-FGFR1, anti-FGFR3, and anti-FGFR4 (1:400, Santa Cruz Biotechnologies), anti-FGFR-2 (1:1000, Sigma), and

anti-glyceraldehyde-3-phosphate dehydrogenase (1:3000, Novus Biologicals). Bound primary antibody was detected using a peroxidase-conjugated goat anti-rabbit secondary antibody (1:2000, Bio-Rad) for 2 h at room temperature. To visualize immunoreactive bands, membranes were exposed to chemiluminescent detection reagents (ECL Plus, GE Healthcare) and images were captured using the ISO400R Kodak Image Station Software (Kodak) and analyzed using the Image J program (Wayne Rasband, NIH). GAPDH was used as the loading control, since previous work has shown that its expression levels do not change after axotomy (Blanco et al, 2008). The protein signal intensities were standardized to the GAPDH intensity, then averaged and normalized against the average control value. The statistical significance was determined using ANOVA and posthoc Tukey or Steel tests. In the results, one asterisk indicates $p < 0.05$, two asterisks $p < 0.01$ and 3 asterisks, $p < 0.001$.

Results

FGF-2 and FGFR are present in RGCs

It was first necessary to confirm that the growth factor and its receptors could be localized to retinal ganglion cells (RGCs). Retinas retrogradely labeled with TDA were sectioned and processed with antibodies against FGF-2, and FGFR1 through 4, resulting in positive colocalization of these proteins in RGCs (Figure 1). FGF-2 was present throughout the control retina, as described previously (Blanco et al., 2000), with most of the staining being localized to TDA-labeled RGCs in the ganglion cell layer (GCL), in subpopulations of cells in the inner nuclear layer (INL) and outer nuclear layer (ONL), and to the glutamate synthetase-positive processes and end-feet of Müller cells (Fig. 1B).

FGF receptor 1 (FGFR1) immunoreactivity was observed in the cytoplasm of most RGCs and in cells at the innermost region of the INL of control retinas. RGC axons were also FGFR1 immunoreactive (Figure 1C). FGFR2 staining was observed in cell bodies of the INL and ONL, and in sublaminae of the IPL of control retinas (Fig. 1D). FGFR2-immunoreactive RGCs in the GCL varied in the intensity of staining, suggesting the presence of subpopulations with different degree of sensitivity or requirement for this receptor. The strongest immunoreactivity was seen in processes of the OPL and in the outer segments of photoreceptors (Fig. 1D).

FGFR3 immunoreactivity was observed in the cytoplasm of the majority of the TDA-labeled RGCs in the GCL, and in some cells of the ONL and INL (Figure 1E). There were also FGFR3 immunopositive Müller cell-like processes in control retinas. FGFR4 was weakly present in RGCs and in a few cells of the INL, ONL, and in Müller cell-like processes (Fig. 1F).

FGF-2 and FGFR immunoreactivities in the retina change after axotomy—An apparent increase in FGF-2 immunoreactivity in cell bodies of the GCL and INL was observed at one week after axotomy (Figure 2 top row), but immunostaining intensities appeared similar to controls at 3 and 6 weeks after axotomy (Fig. 2 top row).

FGFR1 immunostaining intensity appeared to be stronger after axotomy, particularly in the GCL (Fig. 2 middle row). This strong GCL staining seemed to be maintained for up to 6 weeks after axotomy. The distribution and intensity of FGFR2 immunostaining appeared to be unaffected by axotomy (Fig. 2 lower row). FGFR3 staining intensity appeared to be stronger for as long as 6 weeks after axotomy, showing a particularly noticeable increase in intensity in RGCs and their axons (Fig. 3 upper row). FGFR4 immunostaining showed little change after axotomy, possibly becoming fainter in the GCL and INL cell body layers (Fig. 3 lower row).

Immunoblot analysis of the retina

FGF-2 antibody labeled three bands; two bands at 22 and 24 kDa, and a lower band at 18kDa (Fig 4A). Our comparison of control and experimental tissues showed that only the 18kDa band showed significant changes in protein levels (data not shown). One week after axotomy, the levels of FGF-2 were increased by 30% compared to controls ($p < 0.01$), but by three weeks the amounts of the protein had decreased back to basal levels (Fig 4B).

The FGFR1 antibody detected a strong band at 130 kDa (Fig. 4A). At one week after axotomy the protein levels were increased by 55% over control values ($p < 0.001$) and a smaller increase (25–30%) persisted at 3 and 6 weeks ($p < 0.001$). FGFR2 was not studied by Western blotting because of the lack of changes after axotomy observed with immunostaining.

The FGFR3 antibody detected several bands of different weight (175, 150 110, and 55 kDa), only one of which (110 kDa) showed statistically significant changes. FGFR3 protein levels showed a large (135%) increase after axotomy ($p < 0.01$), which appeared to decline somewhat at 3 weeks, but by 6 weeks was increased further to almost 4 times control values ($p < 0.001$). FGFR4 antibody detected a protein at 176 kDa, which declined significantly at one and three weeks after axotomy to 75% of control values ($p < 0.05$), but then recovered to control values at 6 weeks (Fig. 4B).

FGF-2 and FGFR immunoreactivity in the optic tectum—In control optic tectum, the majority of tectal neurons were FGF-2 immunoreactive, as were the occasional blood vessel (Fig. 5 top row). A general decrease in the overall staining intensity of cells was observed at one week after axotomy, but at 3 and 6 weeks there was no obvious difference from controls (Fig. 5 top row).

FGFR1 immunostaining was present in axonal processes within the retinorecipient layer 9 of control tecta; these probably represent RGC axons. (Figure 5 center row) Moderately stained processes and subpopulations of cells in layers 2, 4 and 6 were also observed in control tecta. Increased FGFR1 immunoreactivity was localized in processes of layer 9, and in many tectal cells of layer 8, 6, 4, 2 and 1 at all times after nerve transection (Fig. 5 center row). As in the retina, moderate FGFR2 immunostaining was present in all cell body layers of the tectum, and this staining was unaltered by axotomy (Fig. 5 center row).

Moderate FGFR3 immunostaining was observed in the cytoplasm of cell bodies scattered throughout the various tectal layers of control tecta. After axotomy there was an apparent increase both in the intensity of staining and in the number of immunopositive cell bodies in retinorecipient layer 9 and layer 8 (Fig. 6 top row), an increase which persisted through 6 weeks after axotomy. FGFR4 immunostaining was fairly weak in all layers of control tecta, and appeared to show a transient increase in intensity at 1 and 3 weeks after axotomy (Fig. 6 lower row).

Immunoblot analysis of the tectum—Bands of similar weight to those obtained in retinal samples were detected with antibodies for FGF-2 and FGFRs in tectal homogenates. In contrast to our results in the retina, FGF-2 protein levels were significantly lower than basal levels shortly after axotomy (Figure 7B; 27% less than control, $p < 0.05$). At three and six weeks post nerve injury, FGF-2 was increased back to values that were not significantly different from controls.

Basal levels for all FGFRs were detected in the optic tectum. By one week, axotomy increased the expression of FGFR1, FGFR3, and FGFR4 above control values (FGFR1: 37% more, $p < 0.001$; FGFR3: 120% more, $p < 0.05$; FGFR4: 60% more, $p < 0.01$) (Figure

7B). FGFR1 and FGFR3 remained elevated over basal levels at three weeks and six weeks; FGFR4 levels exhibited a return to control values at six weeks. No significant alterations in FGFR2 expression were determined after axotomy in the optic tectum (data not shown).

Discussion

Previously, our published observations demonstrated that exogenously applied FGF-2 induced a beneficial effect on the long term survival of axotomized RGCs in the adult frog retina (Blanco et al., 2000). However the role of endogenous FGF-2 and its receptors in the response to injury have not been investigated. The object of the present study therefore is to determine the changes in the distribution of this growth factor and its receptors in the retina and its main target, the optic tectum, after optic nerve transection.

FGF-2 is upregulated in the retina after axotomy

We have shown here FGF-2 localization in retinal ganglion cells, in subpopulations of cells in the INL and ONL, and in Müller cell-like processes of control adult frog retina. This is in accordance with what other studies have described in the rat retina: FGF-2 is mainly present in retinal ganglion cells and in Müller cells (Chu et al., 1998; Xiao et al., 1998; Walsh et al., 2001; Yu et al., 2004; Valter et al., 2005). Interestingly, significantly higher endogenous FGF-2 levels have been reported in the adult rat retina compared to the developing animals (Bugra and Hicks, 1997). RT-PCR analysis of retinal tissue from adult zebrafish resulted in the detection of the transcripts for FGF-2 and all four receptors, but no FGF-2 labeling was observed when in situ hybridization of the retina was performed (Hochmann et al., 2012). It has been suggested that constitutive expression of FGF-2 in the retina is relevant for retinal homeostasis, since disruption of its signaling provokes rapid photoreceptor degeneration (Rousseau et al., 2000), and increases activation of caspase-3 in cells of the ONL and INL (Hochmann et al., 2012).

Axotomy transiently increased FGF-2 levels in the retina (1 week after nerve cut), and immunostaining suggests that this increase is mainly localized to cells of the ONL and GCL. Only 16% of the cells in the GCL of the frog retina are amacrine cells (Scalia et al., 1985), and TDA labeling confirms that the majority of the FGF-2-immunoreactive cells in the GCL are ganglion cells. Increased FGF-2 immunoreactivity and mRNA expression has also been shown in the rat retina just hours after retinal detachment (Ozaki et al., 2000) and mechanical injury to the eye (Wen et al., 1995) respectively. Elevated FGF-2 mRNA levels have been demonstrated after optic nerve section in rats (Valter et al., 2005).

Changes in FGF-2 after axotomy have been shown in other areas of the nervous system. For example, FGF-2 mRNA expression was increased after axotomy in the sympathetic superior cervical ganglion (Klimaschewski et al., 1999). Upregulation of FGF-2 mRNA and protein levels, and also increased number of FGF-2 expressing neurons, were found in the dorsal root ganglion after axotomy (Ji et al., 1995). Thus, it appears that upregulation of this growth factor could be a common response of injured nervous tissue; this may result in the promotion of survival directly, or indirectly via the induction of protecting molecules and downregulation of apoptosis mediators (Ríos-Muñoz et al., 2005).

Previous reports of increased FGF-2 levels after injury have stated that this upregulation is transient, as early as hours (Ozaki et al., 2000; Valter et al., 2005) and sustained as long as two weeks (Guillonnet et al., 1998; Lonngren et al., 2006). This transient upregulation of FGF-2 in our model might be sufficient to induce the regenerative capabilities inherent to the frog. In 2006, our lab demonstrated that blocking FGFR1 in the optic nerve axons significantly reduced axotomy-induced upregulation of BDNF and TrkB expression, and phosphorylated CREB and ERK immunoreactivity (Soto et al., 2006), thus demonstrating the

importance of FGF-2 signaling in promoting the expression of known survival-promoting substances.

FGFRs are differentially regulated after optic nerve transection in the frog retina

FGFR1—As reported previously (Blanco et al., 2000), FGFR1 is normally present in frog RGCs and their axons. FGFR1 presence in retinal cells has been reported in the past. FGFR1 was detected in retinal Müller cells, pigment epithelium, photoreceptors and processes of the OPL in the rat retina (Guilloneau et al., 1998; Yu et al., 2004), and in the GCL, INL of the adult zebrafish (Hochmann et al., 2012), pig (Kinkl et al., 2003), and fetal and adult primate retinas (Cornish et al., 2004). In fetal monkey, strong FGFR1 labeling of axonal bundles of the nerve fiber layer was described (Cornish et al., 2004). FGFR1 expression has also been localized in retinal axon growth cones in the developing visual system of *Xenopus* frogs (McFarlane et al., 1995), and in vivo transfection with a dominant negative FGFR1 caused many axons to avoid entering the optic tectum (McFarlane et al., 1996). The latter findings support the importance of FGF-2 signaling in axonal guidance and correct target recognition.

We found that FGFR1 protein and the intensity of immunoreactive cells in the retina were significantly increased shortly after axotomy (1 week) in the frog retina. Higher than normal levels were sustained at time points where regeneration is proceeding. FGFR1 mRNA increased after mechanical injury to the eye in rats (Wen et al., 1995), and after light-induced retinal injury (Guilloneau et al., 1998). Geller and colleagues showed elevated FGFR1 phosphorylation just minutes after retinal detachment in both cats and rabbits (Geller et al., 2001). FGFR1 was upregulated in ONL somas 1 month after optic nerve damage in rats (Valter et al., 2005), and just 12 hours after ischemia (Lonngren et al., 2006). Although FGF-2 is capable of binding all four known high affinity FGF receptors, FGFR1 has been classified as its primary binding target (Lee et al., 1989; Ornitz et al., 1996), and has the strongest kinase activity and thus is the most potent activator of downstream pathways (Raffioni et al., 1999; Dailey et al., 2005; Yamagishi and Okamoto, 2010).

FGFR2—FGFR2 staining of cells in the INL and GCL has been shown in the adult retina of monkeys (Cornish et al., 2004); and expression of this receptor in these same retinal layers, but also including the ONL during development. In the adult rat retina, this receptor was more prominent in retinal inner nuclear layers and in pigment epithelium (Fuhrmann et al., 1999). Our results showed that the immunostaining pattern of FGFR2 appears not to change after optic nerve transection or during regeneration, and there is no evidence from the literature that FGFR2 has any role in survival or regeneration of RGCs.

FGFR3—FGFR3 immunoreactivity has been observed in all plexiform layers, RGCs, photoreceptors and Müller glial cells of both developing and adult primate retina (Cornish et al., 2004). Expression of FGFR3 has also been reported in subpopulation of cells in the INL of the adult zebrafish (Hochmann et al., 2012), in amacrine and RGCs in adult pig retinas (Kinkl et al., 2003), and in RGCs, INL, and IPL of adult and post-natal rat retina (Cinaroglu et al., 2005).

In the frog retina, FGFR3 was greatly increased after axotomy, reaching maximum levels at six weeks, the time when retinal axons are reconnecting to the optic tectum. Significantly higher FGFR3 transcript levels were reported in ischemic rat retina (Ulrika et al., 2006), and also during the development of the nigrostriatal system (Ratzka et al., 2011). RGC survival in vitro is promoted by FGFR3 signaling, mediated via FGF-9 binding (Kinkl et al., 2003). It is possible that the very large increase in FGFR3 in frog RGCs after axotomy is part of their

survival mechanism, but in this case is also mediated via FGF-8, which we have preliminary evidence is also present in the retina.

FGFR4—In control retinas, low amounts of FGFR4 were found in cells of GCL, INL, ONL and in processes of Müller cell-like processes. Similar observations were reported in adult primates (Cornish et al., 2004), pigs (Kinkl et al., 2003), and in rats (Fuhrmann et al., 1999). We show that this receptor is downregulated early after optic nerve transection, and that its basal levels recuperate during regeneration. Analysis of FGFR4 mRNA levels following transient ischemia in rat retinas showed significant reduction just hours after the insult, retaining more normal levels after two weeks (Lönngren et al., 2006).

Axotomy transiently downregulates FGF-2, whereas FGFRs are increased in the optic tectum

The optic tectum, a visual center for vertebrates, receives topographically ordered visual inputs from the retina in the superficial layers and sends motor outputs to the premotor reticulospinal system in the hindbrain (Scalia, 1976).

Very little data is available on FGF-2 and FGFR expression in the optic tectum. Our results show that both FGF-2 and its receptors were normally present in tectal neurons and that axotomy altered their levels. Optic nerve transection transiently decreased FGF-2 in the contralateral tectum, whereas FGFR1, FGFR3, and FGFR4 were upregulated just one week after nerve injury, and FGFR2 showed no change. FGFR-1, FGFR-3, and FGFR-4 have been described in the developing optic tectum of *Xenopus* (Atkinson-Leadbetter et al., 2010). In addition, FGFR-1 and FGFR-2 expression was localized in neuroepithelial tissue anterior to the developing RGC axons reaching the optic chiasm, which could be important for correct optic tract development.

No retinal axons are present at 1 week after axotomy, the time when FGF-2 is reduced in the tectum, and we have shown that RGC axons are re-entering the tectum between 3 and 6 weeks after axotomy (Soto et al., 2006a). This might suggest that the lower FGF-2 levels are simply due to a loss of FGF-2-containing axons; however, the immunostaining shows that only a very small proportion of the total tectal FGF-2 is present in retinal axons, so this decrease must instead be due to a down-regulation of FGF-2 synthesis by tectal neurons. During development, low tectal FGF levels appear to be crucial for incoming retinal axons to recognize the tectum as their target (McFarlane et al., 1995; Webber et al., 2003), so it is possible that the downregulation of FGF-2 synthesis after axotomy makes the tectum more conducive to reinnervation. As axons re-enter the tectum at 3–6 weeks, FGF-2 levels appear to recover, again probably not from the FGF-2 content of the axons themselves, but more likely because of upregulation of synthesis by tectal neurons in response to contacts or signals from the returning axons. It should be noted that, even though many axons have entered the tectum at 6 weeks, functional recovery is not complete before 14 weeks (Singman and Scalia, 1991).

FGFR1 and FGFR3 and, to some extent, FGFR4 levels are increased in the tectum at 1 week after axotomy and, unlike FGF-2, remain high at 3 and 6 weeks. This discrepancy in timing makes it less likely that upregulation of the receptors is simply a direct response to lowered FGF-2 levels alone. However, we cannot rule out possible interactions between other FGF ligands with FGFR-3 receptor. In fact, it has been previously demonstrated that FGF-8 promotes cerebellar development by repressing FGFR3 expression (Liu et al., 2003). In addition, FGF-9 induced survival effect on adult pig RGCs is thought to be via FGFR-3 activation (Kinkl et al., 2003). In fact, we have preliminary evidence that FGF-8 is present in frog tectal neurons, so future experiments should be directed towards finding out if its levels are also altered after axotomy.

Conclusions

Cutting the adult frog optic nerve causes an increase in FGF-2 in the retina while reducing it in the optic tectum. These changes are consistent with its stimulatory effects on RGC survival and its inhibitory effects on target recognition. The larger and more prolonged upregulation of its receptors, FGFR1 and FGFR3, suggests that other members of the FGF family may also play important roles during reconnection after nerve injury.

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Research Highlights

Damage to the optic nerve increases FGF-2 levels in the retina while reducing it in the optic tectum

Axotomy induces a prolonged upregulation of FGFR1 and FGFR3 in both retina and tectum

Axotomy decreases FGFR4 in the retina but increases it in the optic tectum

Figure 1. FGF-2 and its receptors in TDA-labeled retinal ganglion cells

In A, and in C–F, TDA labeling is shown in red, antibody staining in green. A. FGF-2 immunoreactivity is localized in TDA-labeled RGCs in the GCL, in some cells of the INL, ONL, and Müller cell-like processes in the control frog retina. B. glutamine synthetase immunostaining (red) delineates the processes and endfeet of Müller glia, which contain FGF-2 immunostain (green). C. Immunostaining for FGFR1 is present in TDA-labeled RGCs and their axons, and some cells of the INL and ONL. D. FGFR2 immunostaining is present in some TDA-labeled RGCs, and throughout the INL and ONL, with strong staining in the OPL. E. Cytoplasmic FGFR3 immunostaining is present in some TDA-labeled RGCs, with staining also in the INL and ONL and Müller glia. F. FGFR4 immunostaining is faint in TDA-labeled RGCs and in the INL, with some staining in the ONL. Scale bar in D: 50 μ m.

Figure 2. FGF-2 and FGFR1 and 2 in the retina after axotomy

First column: control; second column: 1 week cut; third column: 3 weeks cut; and fourth column: 6 weeks cut. Top row: FGF-2 immunostaining is increased in RGCs in the GCL and in inner cells of the INL at one week after axotomy. Center row: FGFR1 immunostaining is increased at in axotomized animals compared to controls, particularly in RGCs and their axons. Lower row: FGFR2 immunostaining shows no noticeable changes after axotomy. Scale bar: 50 μ m.

Figure 3. FGFR3 and FGFR4 in the retina after axotomy

First column: control; second column: 1 week cut; third column: 3 weeks cut; and fourth column: 6 weeks cut. Top row: FGFR3 immunostaining shows a large increase after axotomy, particularly in the cytoplasm of RGCs in the GCL and in cells of the INL. Staining is strongest at 6 weeks after axotomy. Lower row: FGFR4 immunostaining becomes somewhat fainter in the RGCs after axotomy. Scale bar: 50 μ m.

Figure 4. Immunoblot analysis of retinal homogenates

Western blot analysis of retinal extracts was conducted using FGF-2 and FGFR antibodies. A significant increase in FGF-2, FGFR1, and FGFR3 protein levels were detected at one week after axotomy, whereas FGFR4 levels were reduced. Maximal FGFR-3 levels were reached at six weeks. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, post-hoc Tukey or Steel tests compared to controls). GAPDH levels showed no significant changes (Control: 12.2 ± 0.8 ; 1w: 12.4 ± 0.6 ; 3w: 13.2 ± 0.6 ; 6w: 12.4 ± 0.4).

Figure 5. FGF-2 and FGFR1 and 2 in the tectum after axotomy

First column: control; second column: 1 week cut; third column: 3 weeks cut; and fourth column: 6 weeks cut. Top row: In controls, moderate FGF-2 immunostaining is present in all cell body layers of the tectum. One week after axotomy there is a decrease in the intensity of staining, particularly in layers 6–9. Staining is back to normal levels at 3 and 6 weeks. Center row: In controls, FGFR1 immunostaining is present in all layers of the tectum, with some horizontal neuronal processes stained, particularly in retinorecipient layer 9. There is a marked increase in FGFR1 immunoreactivity in the tectum after axotomy, despite the loss of axons in layer 9 at one week cut. Lower row: Moderate FGFR2 immunostaining is present in all layers of the tectum, and shows no discernible changes after axotomy. Scale bar in A: 100 μ m.

Figure 6. FGFR3 and FGFR4 in the tectum after axotomy

First column: control; second column: 1 week cut; third column: 3 weeks cut; and fourth column: 6 weeks cut. Top row: In controls, FGFR3 moderate immunostaining is present in the cytoplasm of many cells throughout the tectum. One week after axotomy there is a marked increase both in the intensity of staining and in the number of cells stained in layers 7–9. This higher level of staining persists through 6 weeks after axotomy. Lower row: Weak FGFR4 immunostaining is present in all layers of the tectum. This shows an increase in intensity at 1 and 3 weeks after axotomy. Scale bar in A: 100 μ m.

Figure 7. Immunoblot analysis of FGF-2 and FGFRs in the tectum

Tectal homogenates of both control and experimental animals were used for Western blot analysis. Data was first expressed as a ratio with obtained GAPDH loading control protein levels and then normalized against control values. FGF-2 levels are reduced at one week after optic nerve lesion and then recover to basal levels at six weeks. FGFRs were all significantly increased after axotomy. FGFR4 returned to control values at six weeks. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, post-hoc Tukey or Steel tests compared to controls). GAPDH levels showed no significant changes (Control: 12.4 ± 0.4 ; 1w: 11.9 ± 0.6 ; 3w: 11.9 ± 0.4 ; 6w: 12.0 ± 0.5).