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Basic fibroblast growth factor treatment partially protects from visual deficits but does not increase retinal ganglion cell survival following controlled optic nerve crush

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Abstract

Lack of trophic support after axonal injury leads to the degeneration of neurons. To study whether the application of trophic factor can improve functional recovery and retinal ganglion cell (RGC) survival after unilateral controlled optic nerve crush injury, we have now treated adult rats intraocularly (i.o.) with basic fibroblast growth factor (bFGF). To monitor visual deficits, rats were trained in a two-choice pattern discrimination test. Immediately after the crush, and on postoperative days 3 and 6, either 1.1 μg recombinant bFGF or phosphate buffered saline (PBS) was injected i.o. Sham-operated controls received intraocular injection of PBS or bFGF. Within the first few days after the crush, all animals showed a loss of discrimination ability which was followed by a significant recovery within 2–3 weeks. Animals treated with bFGF had a significantly smaller initial deficit and thus recovered earlier compared to PBS controls. Retrograde RGC death was evaluated using retrograde HRP-tracing technique, but bFGF-treatment had no neuroprotective effect. Thus, the behavioral effects of bFGF could not be related to neuroprotection of RGCs and therefore other mechanisms may have to be considered.

1. Introduction

In higher mammals, most retinal ganglion cells (RGCs) degenerate and subsequently die after injury of either the optic nerve or primary visual centers. It is assumed that the death of RGCs is at least in part due to disruption of the supply of trophic factors from target tissues such as the lateral geniculate nucleus of the thalamus (LGN) and the superior colliculus (SC) [7,17,26]. In spite of a general and massive cell death after axotomy or nerve crush [33,39], some RGCs survive axotomy for periods up to several months [1,2,39]. Moreover, it has been shown that surviving retinal ganglion cells can regrow axons within the retina [8,27,32], although in most cases the axonal growth is abortive [19,27]. In the presence of peripheral nerve grafts, axonal regrowth and cell survival can be significantly increased [4,22,40]. It is assumed that trophic products of Schwann-cells, such as nerve growth factor (NGF), play an important role in permitting or supporting cell survival and axonal growth of both peripheral and central neurons [4,24]. Recently, it has been shown that trophic factors not only prevent naturally occurring cell death during development [7] but also increase survival and axonal outgrowth of adult central neurons both in vitro [5,23] and in vivo [3,10,16,20,31]. Among these trophic factors, NGF and the family of fibroblast growth factors (FGF) have received most attention. Treatment with FGF is known to improve the survival rate of different neuron types both in vitro [14,21,25,29] and in vivo [3,9,12,36]. This is also true for RGCs as shown in vitro [5] or in vivo [38] following axotomy.

This trophic action of bFGF on RGCs as documented in several investigations suggests that additional exogenous trophic support might also improve func-

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tional outcome after graded crush of the optic nerve. Previous findings of our laboratory add evidence to this proposal as this neurotrauma model has proven useful to study functional recovery [11], and another pharmacological treatment, using GM_1 gangliosides, has already shown to be able to improve functional recovery following optic nerve injury [35]. The goal of the present study was therefore to assess the efficacy of basic fibroblast growth factor (bFGF) treatment on anatomical and behavioral parameters of neuroprotection after such injury. The present investigation was conducted at a time when we already knew that RGCs degenerate while behavioral performance increased [33,34].

2. Materials and methods

2.1. Surgery and treatment

At the age of 4–5 weeks, the left optic nerve was transected in all rats under general anaesthesia using chloralhydrate (7% solution; 420 mg/kg, intraperitoneally, i.p.). Local anaesthesia of the eye was performed as well using a drop of proxymetacainhydrochlorid (Chibro-Kerakain, CHIBRET). To expose the optic nerve, the conjunctiva lateral to the cornea was cut under a binocular operating microscope (Wild, Typ 308700). After separation of the retractor bulbi muscle, the optic nerve was identified and cut with micro scissors near the eyeball. At 14–16 weeks, the contralateral nerve was also crushed. To do this, the nerve was carefully exposed (same procedure as above), leaving the dura intact. Care was taken not to injure the optic nerve or to damage the retinal blood supply. A 'mild' crush was then made by applying pressure on the optic nerve for 30 s using a special modified cross-action forceps [34] 2–4 mm distal to the eye ball. The 'mild' crush was defined as that force which allows spontaneous functional recovery to occur. It is achieved by setting the jaws of the forceps 0.2 mm apart in 'rest position'. After completion of optic nerve surgery, an antibiotic eye ointment (oxytetracyclin-prednisolon, JENAPHARM) was applied to prevent inflammation in each case. Control animals ($n = 5$) received sham operation only.

Immediately after the optic nerve crush, the animals received an intraocular (i.o.) injection of 2 μl phosphate buffered saline (PBS) containing 1.1 μg of bFGF (recombinant human fibroblast growth factor-basic, Preprotech Inc.; $n = 6$) or 2 μl PBS alone ($n = 4$) using micropipettes attached to a microdispenser (Drummond Model 105). On postoperative days 3 and 6, these injections were repeated under halothane inhalation anaesthesia. Control animals also received i.o. injections of either PBS or bFGF.

2.2. Behavioral assessment: pattern discrimination task

The group identity of all rats was coded and treatment conditions were thus unknown to the experimenter. After a 7-day handling period, animals were trained in the test until they reached an average of at least 85% correct choices. Thereafter, all animals underwent surgery. Water deprivation continued until the end of the behavioral testing at day 21 post-lesion.

Using a similar testing apparatus as described by Sautter and Sabel [34], patterns were presented by a computer screen and the rats had to discriminate between a flickering bar-pattern screen, with five black and white stripes and a grey screen. The spatial frequency of the pattern was 0.17 c/dg (calculated on the basis of the distance between the translucent door and the stimulus) and the flickering frequency was 1.5 Hz; both stimuli had the same luminance. During testing sessions, the room was dimly illuminated.

2.3. Testing procedure

The rats were first accustomed to the test box and then trained to leave the start box and to drink water under the pattern screen. Within 1–2 weeks, rats thus learned to obtain water-reward at the spout which was located beneath the pattern. During this period, most rats reached a criterion of > 85% correct choices. Rats that did not reach this criterion were excluded from the study prior to randomization.

In each trial, rats were first placed into the start box. After 2–3 s the grey guillotine door was opened and the rats could see the stimulus screen. After an additional 3 s, the translucent door was opened as well, whereupon the rats had to run towards the pattern-screen to obtain
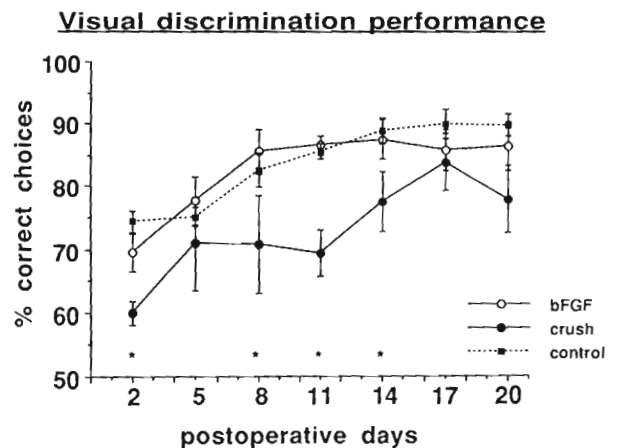

Visual discrimination performance

Fig. 1. Pattern discrimination performance after mild optic nerve crush as indicated by percent correct responses. The data are shown as 3-day blocks (the number on the x-axis corresponds to the postoperative day at the middle of each 3-day block). bFGF treated animals performed significantly better as soon as behavioral testing started (mean ± SE, *$P < 0.05$).

water reward (S +). The side on which S + was presented was determined randomly by the computer (no more than 3 consecutive presentations on the same side). The grey screen was S –. According to this procedure, each rat performed 30 trials a day in one continuous session each. After completion of this pre-training, the rats were randomly assigned to the treatment, and 2 days following optic nerve surgery, behavioral testing commenced. The groups which resulted from the randomization procedure did not differ in their pre-crush performance (data not shown).

2.4. Morphological studies

Upon completion of behavioral testing, retrograde transport of horseradish peroxidase (HRP) was used to quantify the number and size of RGCs in all rats. We also examined the retinae of additional rats ($n = 33$) which received the same treatment as those used in the behavioral test. To do this, rats were first anaesthetized with 420 mg/kg chloralhydrate (i.p.) and placed in a stereotaxic frame (Stoelting, USA). The neocortex overlying the tectum contralateral to the treated eye was exposed by removing a rectangular piece of bone from the skull. A syringe (Hamilton 701 RN, 26S gauge, taper needle) was then filled with 45% HRP (10 μl Sigma type VI or VI-A) dissolved in a 2% aqueous solution of dimethylsulfoxide (DMSO) which was then attached to the syringe holder of the stereotaxic frame. For easier penetration of the injection needle, the dura was opened above the injection site. To expose all optic nerve terminals to HRP, 0.5–1.0 μl of HRP was injected unilaterally into the SC at each of 7 evenly distributed sites according to the coordinates which were determined by a brain atlas. After a slow injection of HRP over a 2 min period, the needle was left in place for an additional 2 min to minimize HRP reflux. Upon completion of the 7 injections, the skin above the skull was sutured and treated locally with antibiotic powder. The survival time after HRP-injection was 48 h.

2.5. Histological preparation

The protocol for the preparation of the retinal whole-mounts was similar to that of Sautter and Sabel [34]. Rats were re-anaesthetized with 420 mg/kg chloralhydrate (i.p.) and perfused transcardially with saline followed by a mixture of 4% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M PBS, pH 7.2. The eye was quickly removed, kept at 4°C and immersed in the fixative while the perfusion of the brain continued for another 10 min. To prepare the wholemount preparations, muscles and connective tissue were removed from the eyeball and the retina was dissected out quickly in a cocktail of PBS/fixative (3:1). The dorsal position of
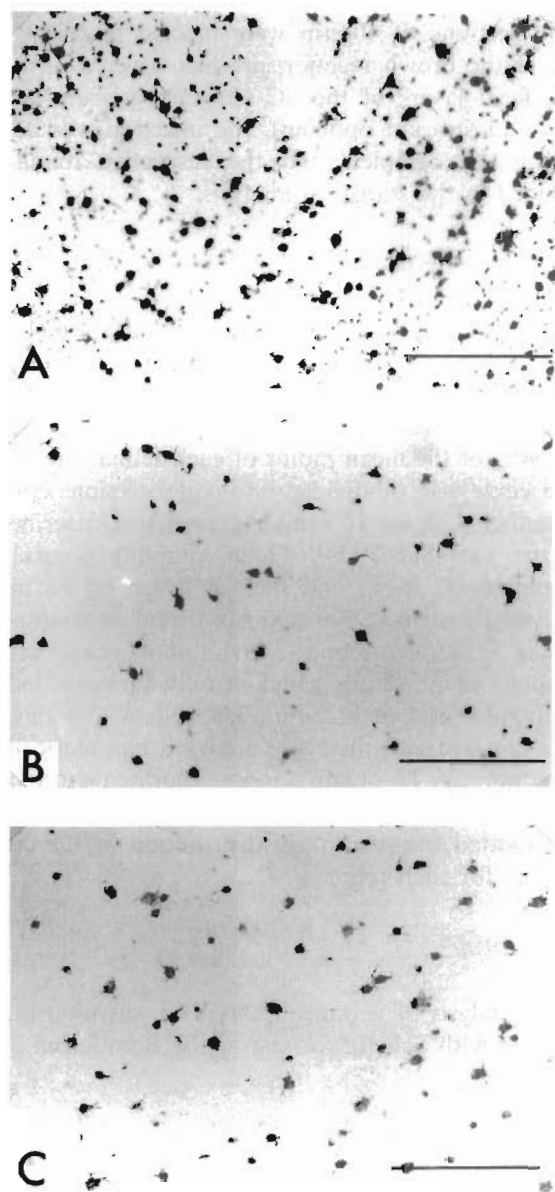

Fig. 2. Representative photomicrographs (32 ×) taken from whole-mounted retinae 25 days after the lesion. A, control; B, crush/PBS; C, crush/bFGF. Bar = 300 μm

the retina was marked by a long radial cut; in most cases 3 additional, smaller radial cuts were made to spread out the retina onto a microscopic glass slide. The retina was cleaned carefully with a fine brush and coverslipped in the fixative. Immediately thereafter, the outline of the freshly prepared retina was drawn at low magnification (10 ×) to obtain a size estimate for the subsequent tissue shrinkage estimate. The histochemical procedure for the HRP-reaction was performed according to Olucha et al. [28]. The fixed whole-mounted retina was then redrawn and the size compared to that of the fresh preparation to calculate tissue shrinkage (the shrinkage was generally in the order of 0.7 ×).

To obtain a criterion of the tectal HRP-injection, brain sections of 40 μm were reacted for HRP [15]; only if the brown reaction product covered all of the superficial layers of the SC (stratum griseum superficiale and stratum opticum), the injection was considered to be complete and the respective retina was included in the statistical analysis.

2.6. Cell analysis

The retinae were drawn at low magnification (1.25 $\times$) under a light microscope (Leitz, LaborLuxS), and concentric circles were added, centering on the optic nerve head. These circles had a radius of 10%, 30%, 50%, 70% and 90% of the mean radius of each retina. Thereafter, each circle was subdivided into equal sections containing either 4, 8, or 12 sampling points as described by Sautter and Sabel [34]. These sampling points were magnified (31.25 $\times$) and then analyzed by an image-analysing system (Leica 500 MC) used in conjunction with a light microscope (Leitz, LaborLuxS). At each sampling point, retinal ganglion cells were counted in a rectangular grid of 122 μm^2. The cells within this area were counted and their size analysed automatically by the computer. To obtain a more accurate picture of the changes within the retinal ganglion cell population, we also plotted the percentage distribution of the cell diameters for each retina.

2.7. Statistics

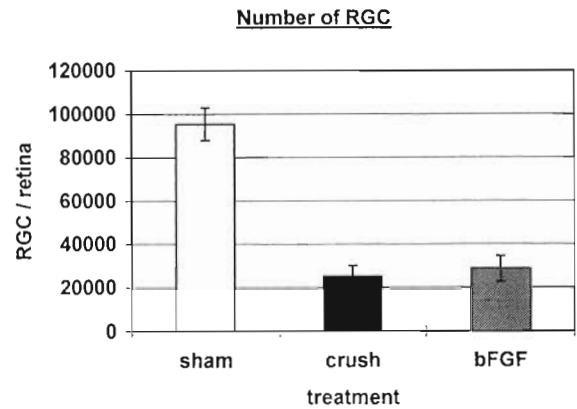An analysis of variance (ANOVA) was used in conjunction with Scheffe's F-test or the Bonnferoni T-test

Fig. 3. The number of retrogradely labelled RGC 25 days after mild optic nerve crush. Cell number was significantly reduced in all lesion groups ($P < 0.0001$, two-tailed) without any difference in the treatment conditions.

for post hoc comparisons to check for statistical differences between groups in the behavioral tests and in the anatomical analysis.

3. Results

3.1. Pattern discrimination performance

The percentage of correct choices in the pattern-discrimination task was used as a behavioral indicator of rat vision. After the optic nerve crush the data were collected daily, but to reduce the variance that results from daily variations in behavioral performance they were pooled into 3-day blocks for subsequent statistical analysis (Fig. 1).
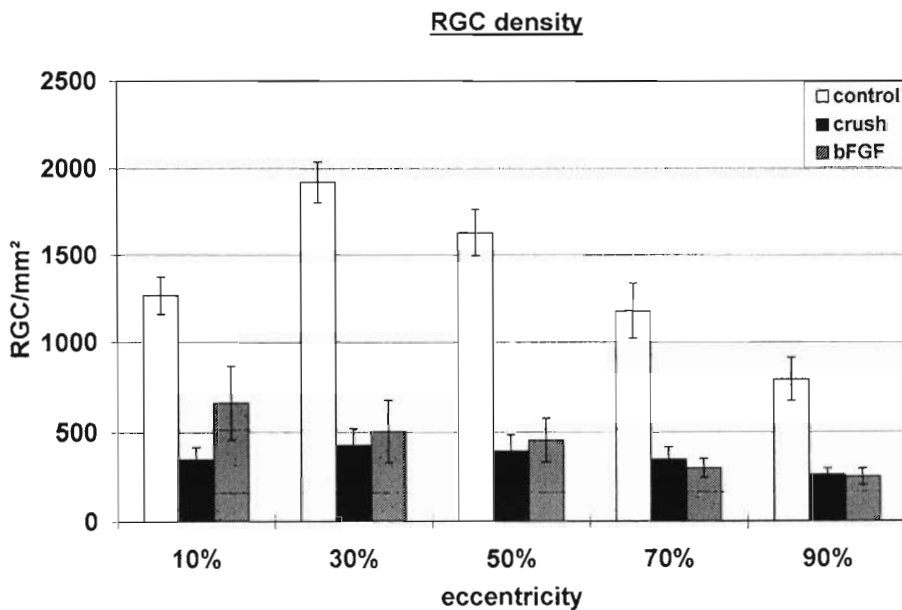

Fig. 4. RGC densities (RGC/mm^2) at defined eccentricities of the retina. At every eccentricity, RGC density in crushed animals is significantly lower compared to uninjured controls ($t = 2.8$, $P < 0.014$, two-tailed).
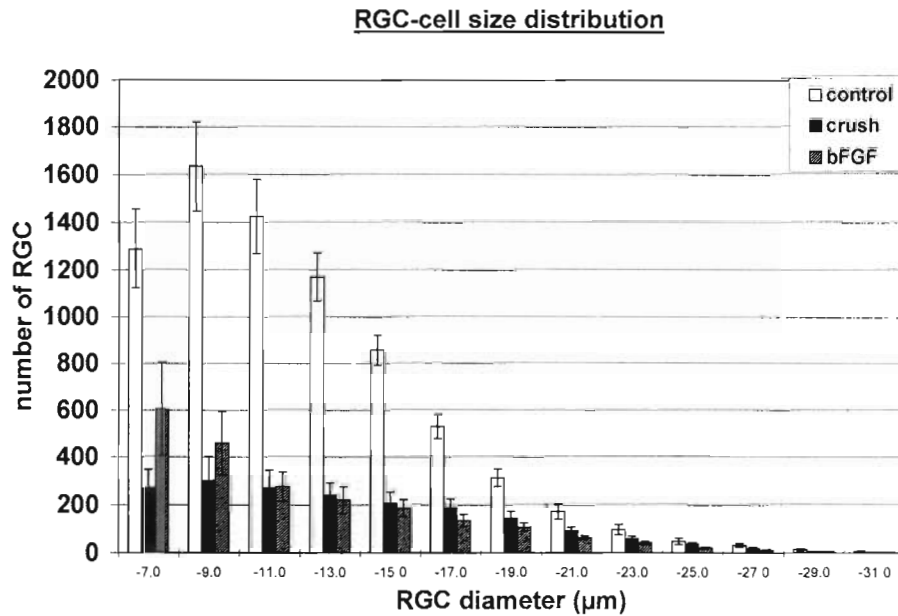
RGC-cell size distribution

Fig. 5. Number of RGC catagorized with respect to their soma diameters. ANOVA [treatment]: $F = 42.80$, $P < 0.0001$; [size]: $F = 68.77$, $P < 0.0001$; [AB]: $F = 21.37$, $P < 0.0001$. For further statistical differences, see Fig. 6.

The sham-operated animals showed some initial deficit which may be attributed to the repeated intraocular injections. Recovery to the pre-surgery levels of > 85% correct choices occurred within 8 days. Because the sham-operated rats treated with i.o. bFGF or PBS injections performed similarly, their data were pooled into a single control group.

Both lesion groups showed a significant improvement over time, with significant treatment effects (ANOVA: factor A [treatment]: $F = 4.38$, $P < 0.04$; factor B [time]: $F = 22.329$, $P < 0.0001$; AxB interaction: $F = 1.966$, $P < 0.08$). At the time points 1, 3, 4 and 5 (3 d-blocks), the bFGF treated animals showed significantly higher vision scores ($t = 1.85$, $P < 0.05$, one-tailed) as compared to the crushed, PBS-treated animals. This effect of bFGF treatment on visually elicited pattern discrimination performance was already seen as soon as behavioral testing started, indicating a behavioral protection rather than enhanced recovery (Fig. 1).

3.2. Anatomical and morphological analysis

RGC counts revealed a significantly larger number of HRP-labelled cells ($95\,493 \pm 7459$, mean $\pm$ SE) in sham-operated controls compared to both lesion groups 25 days after surgery (crush/PBS: $22\,771 \pm 3649$; $t = 8.1$, $P < 0.0001$; crush/bFGF: $28\,473 \pm 5956$; $t = 6.8$, $P < 0.0001$; two-tailed). There was no significant difference between the two treatment conditions (Figs. 2 and 3). This result is also reflected in the comparison of the RGC densities. The analysis revealed an average density of 1326 ± 104 RGC/mm^2 (mean $\pm$

SE) in sham operated controls ($n = 11$). After mild crush, an average number of 348 ± 69 were counted ($n = 13$; $t = 8.1$, $P < 0.0001$, two-tailed), after mild crush plus bFGF treatment, 395 ± 83 RGC/ mm^2 ($n = 9$; $t = 6.8$, $P < 0.0001$, two-tailed) were counted. There was no statistically significant difference between the bFGF-treated and the untreated group. There was also no statistical difference between the crush/PBS and crush/bFGF group in RGC densities at any of the different eccentricities (Fig. 4).

Analysis of the cell diameters of the RGCs in each treatment-group revealed a significantly higher number of RGCs with large diameter (15–25 μm; for statistics, see figure legend) in the crush/PBS treated group compared to controls (Figs. 5 and 6), an effect which was not seen in bFGF-treated animals. Animals which received bFGF-treatment had a slightly, but not significantly, higher number of RGCs with small soma diameters (4.9–7.0 μm, n.s.) (Fig. 6). Whether these changes are important for the functional recovery or a specific effect of bFGF will be addressed in future studies.

4. Discussion

In agreement with previous observations [11,33,34], the present study demonstrates that a mild crush of the optic nerve causes a marked decrease in the performance of visual tasks in rats, which is followed by a functional recovery within about 17 days. I.o. injection of bFGF causes a significant reduction of the initial behavioral deficit. In contrast to the neuroprotective

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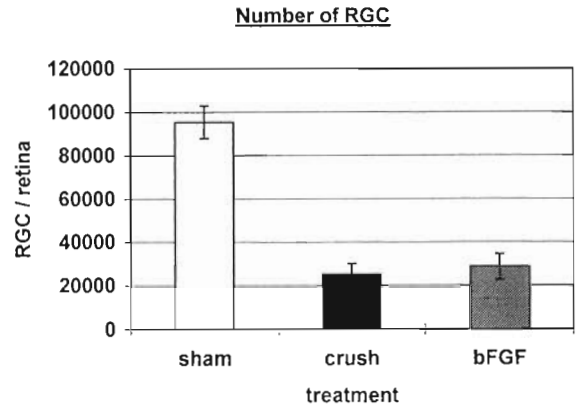
Number of RGC

Fig. 3. The number of retrogradely labelled RGC 25 days after mild optic nerve crush. Cell number was significantly reduced in all lesion groups ($P < 0.0001$, two-tailed) without any difference in the treatment conditions.

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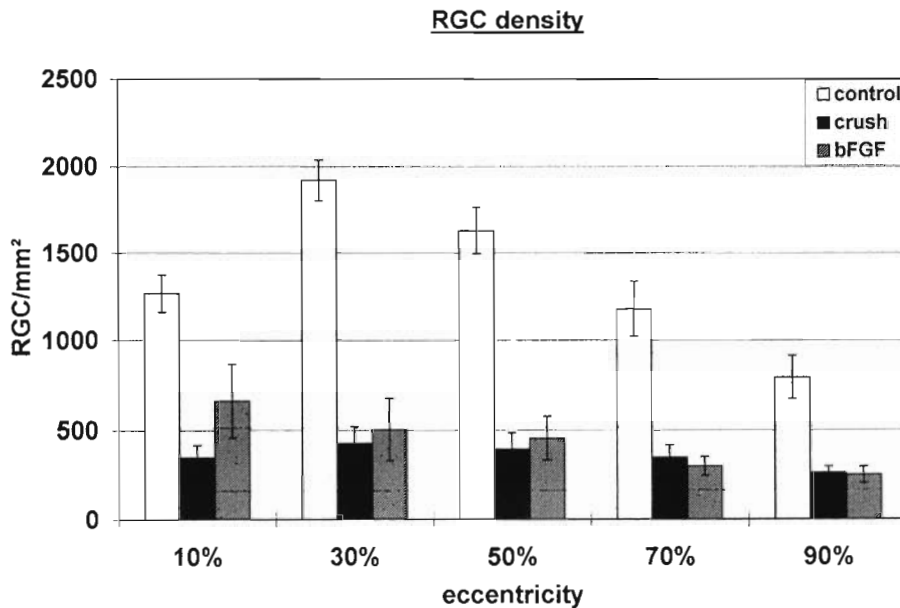
RGC density

Fig. 4. RGC densities (RGC/mm^2) at defined eccentricities of the retina. At every eccentricity, RGC density in crushed animals is significantly lower compared to uninjured controls ($t = 2.8$, $P < 0.014$, two-tailed).

functionally intact or just survive morphologically, as in the Villegas-Perez et al. study [41], and it is not yet clear whether FGF may also affect cells which are connected to their target. Our data indicate that FGF does not affect the number of connected cells but may influence either their function or alter the deafferented target downstream.

One question which was not examined in the present study, but which needs to be addressed in the future, concerns the possible role of the target structure. It is possible that functional recovery may be mediated to a large extent by the target structures. Recent observations of our group using the 2-DG-technique support this view, as local metabolic activity is restored within a similar time course as is recovery of behavioral function [35]. Our present result of behavioral improvement despite the absence of morphological protection in the retina is consistent with this observation, especially if one considers that bFGF is internalized into the cell body and anterogradely transported by adult rat retinal ganglion cells [13]. It is thus possible that the additional trophic support by i.o. injection of bFGF affects downstream structures such as the LGN or the SC. This anterograde (or perhaps transsynaptic) action of bFGF might account for the reduction of the initial deficit we have seen in the present study, a hypothesis which needs to be evaluated more thoroughly in future studies.

Acknowledgements

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