Neuroscience 148 (2007) 53–64

SURVIVAL AND AXONAL REGENERATION OF OFF-CENTER RETINAL GANGLION CELLS OF ADULT CATS ARE PROMOTED WITH AN ANTI-GLAUCOMA DRUG, NIPRADILOL, BUT NOT BDNF AND CNTF

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Abstract—OFF-center retinal ganglion cells (RGCs) occupy a smaller proportion than ON RGCs when RGCs regenerate axons into a transplanted peripheral nerve. We examined whether the regeneration ability of OFF RGCs in adult cats was promoted when the numbers of regenerating RGCs were increased with brain-derived neurotrophic factor (BDNF)+ciliary neurotrophic factor (CNTF)+forskolin (BCF) or 3,4-dihydro-8-(2-hydroxy-3-isopropylamino)-propoxy-3-nitroxy-2H-1-benzopyran (nipradilol), an anti-glaucoma drug. ON or OFF RGCs were morphologically determined on the basis of their dendritic ramification in the inner plexiform layer using computational analysis. In the normal intact retina the ratio of ON and OFF RGCs (ON/OFF ratio) was 1.25 (55%/44%); whereas, it was 2.61 in regenerating RGCs with saline injection (control) 6 weeks after peripheral nerve transplantation. Estimated numbers of regenerating ON and OFF RGCs were 2149 and 895, respectively. An injection of BCF increased only numbers of ON RGCs into 5766 (2.7-fold to control) but not that of OFF RGCs, *n*=858. Nipradilol increased both estimated numbers of ON (11,518, 5.4-fold to control) and OFF RGCs (7330, 8.2-fold to control). In the retinas with optic nerve (OpN) transection and intravitreal saline-, BCF- or nipradilol-injection, numbers of ON and OFF RGCs surviving axotomy showed similar trend to that in regenerating RGCs. Thus, nipradilol promoted the survival and regeneration abilities of both of ON and OFF RGCs whereas BCF only did the abilities of ON RGCs. The distribution of tropo-myosin-related kinase B, BDNF receptor, was sparser in the outer two thirds of inner plexiform layer. The lower surviving ability of OFF-RGCs may be attributed in part to the distribution.

Key words: optic nerve regeneration, optic nerve transection, OFF-center cell, peripheral nerve transplantation, neuroprotection.

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Abbreviations: BCF, brain-derived neurotrophic factor+ciliary neurotrophic factor+forskolin; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CNTFR-α, alpha subunit of ciliary neurotrophic factor receptor; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; IGF-1, insulin-like growth factor-1; INL, inner nuclear layer; IPL, inner plexiform layer; LY, Lucifer Yellow; NAB, not alpha/beta (cell); nipradilol, 3,4-dihydro-8-(2-hydroxy-3-isopropylamino)-propoxy-3-nitroxy-2H-1-benzopyran; NO, nitric oxide; OpN, optic nerve; PBS, phosphate-buffered saline; PN, peripheral nerve; RGC, retinal ganglion cell; trkB, tropo-myosin-related kinase B.

Transection of the optic nerve (OpN) in adult mammals results in the death of retinal ganglion cells (RGCs), but some RGCs survive axotomy and regenerate their axons when a peripheral nerve (PN) is transplanted to the transected OpN (So and Aguayo, 1985; Politis and Spencer, 1986; Watanabe et al., 1991) or the pathway is bridged with self-assembling peptide nanofiber scaffold (Ellis-Behnke et al., 2006). Since the number of RGCs with regenerated axons is only 2%–5% of the total population (Watanabe et al., 1993; Watanabe and Fukuda, 2002), trials have been done to increase the number of regenerating RGCs, such as intravitreal injections of brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) (Cui and Harvey, 2000; Cui et al., 2003), or an analog of cyclic AMP (Cui et al., 2003; Monsul et al., 2004).

An injection of the combination of BDNF+CNTF+forskolin (BCF), an accelerator of adenylate cyclase, increases the number of regenerated RGCs by threefold (Watanabe et al., 2003). Recently we have reported that 3,4-dihydro-8-(2-hydroxy-3-isopropylamino)-propoxy-3-nitroxy-2H-1-benzopyran (nipradilol), an anti-glaucoma drug having functions of a nitric oxide (NO) donor and beta adrenoreceptor blocker, greatly increases the number of regenerating RGCs when injected into the cat vitreous (Watanabe et al., 2006). The increases are 4.0-fold in total RGCs, 7.1-fold in beta cells, 4.3-fold in not alpha/beta (NAB) cells at week 6, respectively, and 3.4-fold in alpha cells at week 4. Then a question arose whether the BCF or nipradilol promotes regeneration of OFF-RGCs, since OFF-center RGCs do not have so effective ability of regeneration as ON-center RGCs (Watanabe and Fukuda, 1997; Miyoshi et al., 1999). In the normal intact cat retina the number of OFF-center RGCs is approximately equal to that of ON-center RGCs (Stone and Fukuda, 1974; Cleland et al., 1975; Peichl and Wässle, 1981; Wässle et al., 1981a,b), hence it is a reasonable assumption that acute vision may be difficult to envisage with smaller numbers of regenerating OFF RGCs.

This morphological study first verified our previous reports that the number of ON-center (sublamina *b*) RGCs exceeded that of OFF-center (sublamina *a*) RGCs by analyzing more regenerating RGCs than in our previous studies (Watanabe and Fukuda, 1997; Miyoshi et al., 1999). Then we examined whether the number of regenerating OFF RGCs increased when the total number of regenerating RGCs was increased with the drugs. We found that the axonal regeneration of OFF RGCs was enhanced in the retina with an intravitreal injection of nipradilol, but not with an injection of BCF. Similarly, OFF RGCs degener-

0306-4522/07$30.00+0.00
doi:10.1016/j.neuroscience.2007.05.042

ated faster after axotomy than ON RGCs, and their vulnerability was rescued with nipradilol but not BCF.

EXPERIMENTAL PROCEDURES

Animals and anesthesia

The treatment of animals was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Institutional Guidelines for Laboratory Animal Care and Treatment. We tried to minimize the number of animals used and their suffering. Table 1 summarizes number of animals that were used in the present study. Thirty cats used in the present study were not overlapped with those in our previous study (Watanabe et al., 2001, 2003, 2006; Maki et al., 2003). Adult cats, 2–3 kg, were sedated with an i.m. injection of 60 mg ketamine, then with a gas mixture of oxygen 1.5 l/min, nitrous oxide 1 l/min and 1–2% halothane. The head was fixed in a stereotaxic head holder (SN-3N, Narishige Scientific Instrument Lab, Tokyo, Japan). The heart rate was stabilized with atropine sulfate, 1 mg/kg, i.p. The electrocardiogram was monitored during surgery.

Labeling of RGCs with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)

The detailed procedures have been previously described (Watanabe et al., 2001, 2003, 2006). To label normal RGCs with DiI (Molecular Probes, Eugene, OR, USA), a 10 μl Hamilton syringe (701N, Hamilton, Reno, NV, USA) with a needle insulated using epoxy resin, tip resistance=2–5 MΩ, was filled with the DiI-suspension at a final concentration of 5 mg/ml, and fixed on an electrode holder. The syringe was positioned vertically at anterior=3.0–9.0 mm, lateral=8.0–9.0 mm, and positioned in the lateral geniculate nuclei and the optic tracts by recording field potentials in response to light flashes. A total of 30–40 μl of the suspension was injected bilaterally through 10–14 tracks into the lateral geniculate nuclei and optic tracts. A suppository containing 15 mg sodium pentobarbital (Wakobital 15, Wakodo Pharmaceutical, Tokyo, Japan) was administered to reduce stress and tremor.

Transection of OpN and PN transplantation

Ten to 14 days after DiI injections, the cats were anesthetized with the gas mixture. Penicillin G (Meiji Seika, Tokyo, Japan), 2×10^5 units in saline, was injected intracutaneously at the beginning of surgery. After removing the bones over the frontal sinus and the orbit, and then also the dorsal ocular muscles, the left OpN was exposed. Using microsurgery scissors and an L-shaped hook, the OpN sheath was cut longitudinally then the OpN pulled up from the sheath and cut at 3–4 mm from the eye. Care was taken not to injure the blood vessels, especially the ophthalmic artery.

The left common peroneal nerve, 40–55 mm, was excised. After the transection of the left OpN, the cut end of the PN was sutured to the stump with nylon thread (10–0 Ethicon, Ethilon, Somerville, NJ, USA) and the other end of the graft was left in the temporalis muscles. The skin over the orbit was closed.

Intravitreal drug injection

A hole was opened with a 26-gauge needle in the sclera posterior to the ora serrata and 10 μl of drug solution or saline (control) was injected into the vitreous using a 10 μl Hamilton microsyringe (701N, Hamilton). Care was taken not to injure the lens and retina.

One milligram of nipradilol (Kowa, Nagoya, Japan) was dissolved in 0.1 ml of 0.1 N HCl. The solution was diluted with 0.9 ml of 0.1 M phosphate buffer, pH 7.4, then 110-fold with saline. When 10 μl of the nipradilol solution was injected into the cat vitreous at a mean volume of 2.7 ml, the nipradilol concentration was 0.1 μM. The solution was freshly prepared at every experiment. The injection was performed 40–90 min prior to the OpN transection (Watanabe et al., 2006).

Two micrograms of BDNF (human recombinant, Sumitomo Pharmaceutical, Osaka, Japan), 1 μg of CNTF (rat recombinant, PeproTech EC, London, UK), and 0.1 mg of water soluble forskolin (RBI, Natick, MA, USA) were dissolved in 10 μl saline, and intravitreally injected immediately after the OpN transection (Watanabe et al., 2003).

Labeling RGCs with regenerated axons

The procedures for labeling RGCs, intracellular injection of Lucifer Yellow (LY) and estimation of total number of regenerating RGCs were described previously (Watanabe et al., 1993, 2003; Maki et al., 2003). The graft was exposed under a surgical scope 2 days before dissection of retinas. Approximately 20 μl of dextran-conjugated Texas Red (molecular weight 10 kDa, Molecular Probes), 10% in saline, was injected into the graft at 5–10 mm from the connection of the optic stump with a 10 μl Hamilton microsyringe.

Intracellular injection of LY

The cats were anesthetized with the gas mixture and fixed in the stereotaxic head holder 10 days after DiI injection (intact retina), 14 days after OpN transection or 6 weeks after PN transplantation when the number of regenerating RGCs reaches plateau (Maki et al., 2003). After the left eyes were enucleated, the cats were killed with an i.v. injection of an overdose of sodium pentobarbital (To-

Table 1. Experimental design and number of animals used for experiments

Experiment	Intravitreally injected drug and amount	Survival	Number of animals
LY injection for intact retina	None	—	3
Neuroprotection			(Total: 8)
Control	Saline, 10 μl	2 wk	3[a]
BCF	BDNF 2 μg, CNTF 1 μg, forskolin 0.1 mg	2 wk	3
Nipradilol	Nipradilol 0.1 μM[b]	2 wk	2[a]
Regeneration			(Total: 15)
Control	Saline, 10 μl	6 wk	9
BCF	BDNF 2 μg, CNTF 1 μg, forskolin 0.1 mg	6 wk	3
Nipradilol	Nipradilol 0.1 μM[b]	6 wk	3
Immunocytochemistry for trkB and CNTFR-α	None	2 h; 2, 4, 7d	4[a]
Total number of animals			30

[a] The right retina was used for normal intact experiments.
[b] Final concentration in the vitreous, average volume=2.7 ml.