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AXONAL REGENERATION OF CAT RETINAL GANGLION CELLS IS PROMOTED BY NIPRADILOL, AN ANTI-GLAUCOMA DRUG

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Abstract—Neurons in the CNS can regenerate their axons in an environment of the peripheral nervous system, but this ability is limited. Here we show that an anti-glaucoma drug, nipradilol, at low concentration led to a four-fold increase in the number of cat retinal ganglion cells regenerating their axons into a transplanted peripheral nerve 4 and 6 weeks after axotomy. Nipradilol also increased the number of three main regenerating retinal ganglion cell types (alpha, beta, not alpha/beta), and enhanced the rate of axonal regeneration of these retinal ganglion cells. Nipradilol is a donor of nitric oxide and an antagonist of alpha-1, beta-1 and -2 adrenoreceptors, and we therefore examined whether one of these pharmacological effects might be more important in promoting axon regeneration. A nitric oxide donor increased the number of regenerating retinal ganglion cells, but not the rate of axonal regeneration. Denitro-nipradilol (nitric oxide-deprived nipradilol) or a nitric oxide scavenger injected before nipradilol increased the number of regenerating retinal ganglion cells but did not promote regeneration rate. Blockade of individual alpha- and beta-adrenoreceptors did not increase the number of regenerating retinal ganglion cells or the rate of regeneration. From these results, it is suggested that nitric oxide plays a crucial role in mediating the effects of nipradilol on axon regeneration and neuroprotection, and the metabolite of nipradilol supports the effects. © 2006 Published by Elsevier Ltd on behalf of IBRO.

Key words: optic nerve regeneration, nitric oxide, neuroprotection, adrenergic receptors, retinal ganglion cells, peripheral nerve transplantation.

In adult mammals, axotomized neurons in the CNS can regenerate their axons when presented with a permissive milieu such as a transplanted peripheral nerve (PN) (David and Aguayo, 1981; Benfey and Aguayo, 1982; see Aguayo, 1985). However, the number of CNS neurons that regenerate their axons into a PN graft is very limited. For example, only 2%–5% of retinal ganglion cells (RGCs) extend their axons into a PN anastomosed to the transected cat optic nerve (OpN) (Watanabe et al., 1993; Maki et al., 2003). With such low numbers it is difficult to envisage restoration of visual function, especially with respect to acuity (Watanabe and Fukuda, 2002). This is in contrast with other pathways such as spinal tracts in which even ≈1% fibers can allow for the recovery of some motor function (Li et al., 1997; Keyvan-Fouladi et al., 2003). Therefore, novel approaches are required to stimulate a greater number of RGCs to regenerate their axons.

Surviving axotomy is the first requirement for RGCs to be able to regenerate their axons (Goldberg and Barres, 2000; Watanabe and Fukuda, 2002), although sometimes survival and regeneration are inversely regulated (Dusart et al., 2005). It is a reasonable assumption that promoting the survival of axotomized RGCs would result in increase of number of regenerated axons. Neuroprotective drugs (neurotrophism), however, are not necessarily specific promoters of axonal regeneration (neurotropism) (Lu et al., 2001). For example, brain-derived neurotrophic factor (BDNF) is a potent trophic factor associated with the survival for RGCs (Heiduschka and Thanos, 2000; Yip and So, 2000), but not with their axonal regeneration, with the exception of some spinal neurons (Lu et al., 2001). We have reported the similar result that a combination of BDNF, ciliary neurotrophic factor, and forskolin increases the number of regenerating RGCs as well as that of surviving RGCs, but does not promote the rate of axonal regeneration of surviving RGCs (Watanabe et al., 2003). Nevertheless, searching for neurotrophic effect is a pertinent approach for exploring whether a drug should be further tested for its neurotropic effect.

Here we investigated the neurotrophic and neurotropic effects of nipradilol 3,4-dihydro-8-(2-hydroxy-3-isopropylamino)-propoxy-3-nitroxy-2H-1-benzopyran, which is clinically used to treat patients with glaucoma in Eastern Asia. Since nipradilol has a neuroprotective effect on axotomized RGCs in rats (Mizuno et al., 2001; Kashiwagi et al., 2002; Nakazawa et al., 2002; Taniai et al., 2002), we first examined whether nipradilol was neuroprotective for axotomized RGCs in adult cats. We then tested whether this drug would enhance the rate of axonal elongation as well as increase the number of regenerating RGCs. We adopted the experimental model involving transplantation of a PN segment to the cut OpN stump, which enables RGCs to extend their transected axons into the PN graft. We show that nipradilol increased the number of regenerating RGCs by four-fold at weeks 4 and 6, and also in-

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Abbreviations: BDNF, brain-derived neurotrophic factorcarboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide potassiumdenitro-nipradilol, 3,4-dihydro-8-(2-hydroxy-3-isopropylamino)-propoxy-3-hydroxy-2H-1-benzopyranDiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FL-D, fluorescein-conjugated dextran; L-NAME, N-omega-nitro-L-arginine methyl ester; LY, Lucifer Yellow; NAB, not alpha/beta (cells); nipradilol, 3,4-dihydro-8-(2-hydroxy-3-isopropylamino)-propoxy-3-nitroxy-2H-1-benzopyran; NO, nitric oxide; NOS, nitric oxide-synthesizing enzymes; OpN, optic nerve; PN, peripheral nerve; RGC, retinal ganglion cell; RH-D, tetramethylrhodamine-conjugated dextran; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside.

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

Experiment	Intravitreally injected drug and amount	Survival (week)	Number of animals	Eyes used for cell count and LY injection
Neuroprotection			(Total: 25)	
Control	None	1	4	Both
Control	None	2	4	Both
Saline	Saline, 10 μL	2	5	Both
Nipradilol	Nipradilol, 10 μM	1	3	Both
Nipradilol	Nipradilol, 0.1 μM	1	4	Both
Nipradilol	Nipradilol, 0.1 μM	2	3	Both
Nipradilol	Nipradilol, 0.01 μM	1	2	Both
Regeneration			(Total: 54)	
Control	None	4	6	Left
Control	None	6	14	Left
Saline	Saline, 10 μL	6	2	Left
Nipradilol	Nipradilol, 1 μM	6	2	Left
Nipradilol	Nipradilol, 0.1 μM	4	4	Left
Nipradilol	Nipradilol, 0.1 μM	6	9	Left
Nipradilol, immediate	Nipradilol, 0.1 μM, <10 min	6	2	Left
Prazosin (alpha-1, -2B)[a]	Prazosin, 10 μM	6	2	Left
Timolol (beta-1)[a]	Timolol, 10 μM	6	2	Left
ICI (beta-2)[a]	ICI-118,551, 10 μM	6	2	Left
SNP	SNP, 100 μM	6	3	Left
DNNP	Denitro-nipradilol, 0.1 μM	6	3	Left
PTIO+nipradilol	Carboxy-PTIO, 100 μM+Nipradilol, 0.1 μM	6	3	Left

[a] Parentheses, type of adrenoreceptors blocked with the drug.

creased the rate of axonal regeneration. We further examined the mechanism by which the drug promoted axonal regeneration. The results suggest that nitric oxide (NO) at low concentration may mediate the effect of nipradilol in promoting axonal regeneration of RGCs in adult cats.

EXPERIMENTAL PROCEDURES

The treatments of animals were 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. Efforts were made to minimize the number of animals used and their suffering. Table 1 summarizes number of animals used in the present study. To reduce the number of experimental animals, only two cats were studied when an injected drug was found not to increase the number of surviving or regenerating RGCs.

Anesthesia of cats

Adult cats, weighing 1.8–3 kg, were sedated with an i.m. injection of 60 mg ketamine, anesthetized with a gas mixture of oxygen 1 l/min, nitrous oxide 0.5–0.8 l/min, and 1–2% halothane, and fixed in a stereotaxic head holder (Narishige SN-3N, Tokyo, Japan). Penicillin (200,000 units, Meiji Seika Pharmaceutical, Tokyo, Japan) was administered intracutaneously at the beginning of surgery. Heart rate was stabilized with atropine sulfate (1 mg/kg body weight) injected intraperitoneally. The electrocardiogram was monitored during surgery.

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

RGCs were labeled with DiI (Molecular Probes, Eugene, OR, USA) using a procedure that has been described previously (Watanabe et al., 2001, 2003). In brief, DiI was dissolved in dimethyl sulfoxide at 100 mg/ml, then suspended with constant agitation by sonication in saline supplemented with Triton X-100, 0.1%, at a final concentration of 5 mg/ml. A 10 μl Hamilton syringe (Hamilton 701N, Reno, NV, USA) with a needle insulated using epoxy resin, tip resistance=2–5 MΩ, was filled with the DiI-suspension, 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 five to seven 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.

OpN transection

Ten to 14 days after DiI injections, the cats were anesthetized with a gas mixture. 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. The skin over the orbit was closed. Seven or 14 days later, both retinae were dissected and used for Lucifer Yellow CH (LY, Sigma, St. Louis, MO, USA) injections.

PN transplantation

Surgical procedures for transplantation of a segment of PN were described previously (Watanabe et al., 1993; Maki et al., 2003). In brief, the left OpN was exposed in the anesthetized cats, and transected 3–4 mm posterior to the eyeball. The left peroneal nerve, 40–55 mm, was excised and the skin was closed. Two markers of nylon thread (Ethilon 10–0, 2860G, Ethicon, Somerville, NJ, USA) were sutured to the PN segment at 10 and 20 mm. The PN segment was anastomosed to the OpN stump with nylon threads (Ethilon 10–0, 2860G). The other end of the PN graft was left blind in the temporalis muscle.