portedly maintain longer survival of axotomized RGCs (Stichel and Müller, 1998; Heiduschka and Thanos, 2000). The second purpose was to systematically analyze which neurotrophic factor enhances the survival of β cells. We also examined the effect of supplementation of forskolin on their survival because the drug facilitates internalization of neurotrophic factors by raising intracellular concentration of cAMP (Meyer-Franke et al., 1995). Finally, we tested whether the intravitreal injections of neurotrophic factors and forskolin increased numbers of RGCs which regenerated axons into a PN graft.

The present study shows that combined intravitreal injections of neurotrophic factors and forskolin most effectively enhance the survival of axotomized cat RGCs, especially β cells. Moreover, the combination significantly increased the numbers of regenerated β cells.

EXPERIMENTAL PROCEDURES

Seventy-five adult cats, weighing 1.8 to 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 (0.8 l/min), and 1 to 2% halothane, and fixed in a stereotaxic head holder (Narishige SN-3N, Tokyo, Japan). The treatment of animals was in accordance with institutional guidelines.

Labeling of RGCs with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

The procedure has been described previously (Watanabe et al., 2001). In brief, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, Eugene, OR) was dissolved in dimethyl sulfoxide at 100 mg/ml, and 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) whose needle was insulated with epoxy resin, tip resistance=2–5 MΩ, was filled with DiI suspension fixed on an electrode holder. The syringe was positioned vertically (anterior, 3.0–9.0 mm, lateral, 8.0 or 8.5 mm) and advanced into the lateral geniculate nuclei and the optic tracts by recording field potentials in response to light flashes. A total of 30 to 40 μl of the suspension was injected bilaterally through five to seven tracks into the lateral geniculate nuclei and optic tracts. A suppository for human infants, containing 15 mg sodium pentobarbital (Wakobital 15, Wakodo Pharmaceutical, Tokyo, Japan), was administered to reduce stress and tremor during recovery.

Optic nerve transection

Surgical procedures for transection of the ON were described previously (Watanabe et al., 2001). In brief, 10–14 days after DiI injections, the cats were anesthetized with the gas mixture. After the bones over the frontal sinus and the orbit and dorsal extracellular muscles were removed, the left ON was exposed. With microsurgery scissors and an L-shaped hook, the sheath of the ON was cut longitudinally and the ON pulled up from the sheath and cut at 3–4 mm from the eye. The skin over the orbit was closed. Penicillin (200 k units, Meiji Pharmaceutical, Tokyo, Japan) was dissolved in saline administered intracutaneously at the beginning and end of the surgery. After 14 days of survival, both retinas were dissected and used for Lucifer Yellow (LY) injections.

Transplantation surgery

Surgical procedures for transplantation of a PN segment were described previously (Watanabe et al., 1993). The left ON was exposed, transected 3–4 mm posterior to the eyeball, as described above. The peroneal nerve of the left thigh, 40–65 mm, was excised and the skin was closed. The nerve was sutured to the ON stump with nylon thread for microsurgery (Ethilon 10-0, 2860G, Ethicon, Somerville, NJ), and the other end of the graft was left in the temporalis muscle. Penicillin (200 k units) was administered intracutaneously at the beginning and end of the surgery.

Intravitreal injection of neurotrophic factors

One of the following neurotrophic factors was dissolved in 50 mM Tris buffer at 0.1 μg/μl: brain-derived neurotrophic factor (BDNF, human recombinant, Pepro Tech, London, England, or Sumitomo Pharmaceutical, Osaka, Japan), basic fibroblast growth factor (FGF2, human recombinant, Pepro Tech), ciliary neurotrophic factor (CNTF, rat recombinant, Pepro Tech), glial cell-derived neurotrophic factor (GDNF, human recombinant, Pepro Tech), nerve growth factor (NGF, human recombinant, Pepro Tech), or neurotrophin 4 (NT-4, human recombinant, Pepro Tech). Immediately after ON transection, a hole was opened with a 26-gauge needle in the sclera posterior to the ora serrata, and then a 10-μl solution of each factor or saline (control) was injected into the vitreous with a 10-μl Hamilton syringe. Care was taken not to injure the lens or retina. In some experiments, water-soluble forskolin (0.1 mg, F-111, RBI, Natick, MA) was added to the solution.

Microelectrodes for intracellular injections

Microelectrodes were made from glass capillaries (GC100F-10, Clark, Reading, England) with a microelectrode puller (Sutter P-97, Novato, CA). The microelectrodes were filled with 2% (w/v) LY CH (L0259, Sigma, St. Louis, MO) in 50-mM Tris buffer, pH 7.4, and used without beveling. Their tip resistances were 150–250 MΩ. Negative current, 5–10 nA, was applied constantly while the electrodes were advanced to RGCs to be injected.

Labeling RGCs with regenerated axons

On day 57 after the PN transplantation, the cats were anesthetized with the gas mixture. The graft was exposed under a surgical scope. Approximately 30 μl of dextran–fluorescein (D-1820; molecular weight, 10,000; Molecular Probes) or dextran–Texas Red (D-1863; molecular weight, 10,000; Molecular Probes), 10% in saline, were injected into the graft at 5–8 mm from the connection of the optic stump with a 10-μl Hamilton syringe. After 3 days of survival, the dissected retinas were used for LY injections.

Intracellular injection of LY

The cats were anesthetized with the gas mixture and fixed on a stereotaxic head holder. The eyes were enucleated, and the retinas were dissected in Ames medium (A-1420, Sigma), supplemented with sodium hydrogen bicarbonate (10.9 g/l), and aerated with a 95% O_2–5% CO_2 gas mixture. The retina was affixed to a thin layer of 30% gelatin in a chamber where the aerated Ames medium was circulated.

In neurotrophic factor injections, somata and processes of labeled RGCs in three regions of the temporal retina, eccentricity ~4 mm, were visualized with LY injections under an ×40 water immersion objective (LUMPlan FL, Olympus, Tokyo, Japan). In peripheral nerve transplantation experiments, two regions in the temporal retinas, eccentricity ~4 mm, and one close to the area centralis were chosen for the injections. LY was systematically injected into 60–90 RGCs in the three regions, and then the proportions of cell types were determined as previously reported (Watanabe et al., 1993). In the paired right retina in neurotrophic injection experiments, the proportions of cell types were also determined in three regions of the temporal retina with the same

procedure. The left and right retinas were fixed with 3% paraformaldehyde in 0.1 M phosphate buffer and mounted in PermaFluor (Immunon, Pittsburgh, PA). An overdose of sodium pentobarbital (3–5 ml, Nembutal, Abbott Laboratories, North Chicago, IL) was intravenously administered after the enucleation.

Acridine Orange labeling

Six retinas with the peripheral nerve transplantation were vitally stained with Acridine Orange (Merck, Darmstadt, Germany). After LY injections, several drops of Acridine Orange, 5 μM in Ames medium, were added to the chamber for 3 min, and then the medium was washed away. The retina was peeled off the gelatin plate, fixed with 3% paraformaldehyde, and mounted in PermaFluor.

Measurement of spatial density in the area centralis

In the ON-cut and neurotrophic factor injection experiments, RGCs labeled with DiI in the area of horizontal 540 μm and vertical 360 μm centered at the area centralis of the right and left retinas were photographed with high-sensitivity negative films (Fujicolor Superia 1600, Fuji Film, Tokyo, Japan) under a G2A filter system (510–560-nm excitation filter, and 590-nm long-pass barrier filter, Nikon, Tokyo, Japan). Labeled RGCs were counted on photographs in the area of 540 μm×360 μm centered at the area centralis at ×250 with an ×2.5 magnifier. The ratios of RGC densities in the left retinas versus those of paired right retinas were regarded as the ratio of total numbers of surviving RGCs in the left to the right retinas (Watanabe et al., 2001).

Estimation of the total number of regenerated RGCs

Dextran–fluorescein or dextran–Texas Red-labeled RGCs were counted in a 480-μm×480-μm area on 310–410 grid points each separated by 1 mm. RGCs whose nuclei were in the area, or either on the upper or left borderline, were counted. The cytoplasm was not used as a border for counting. This counting covered 23% of the whole retina. The cell density in 1 mm^2 was calculated, and the total cell number was estimated as the summation of the cell number in each 1-mm^2 square.

Estimation of ratios of each cell type in the ON-transected retinas

The procedure to estimate the numbers of RGCs was described previously (Watanabe et al., 2001). In brief, the ratios of the numbers of surviving α (Rα), β (Rβ), and NAB cells (Rn) on day 14 to the numbers in the intact retinas are given as:

$$R\alpha = Rt \times P\alpha l / P\alpha r$$

$$R\beta = Rt \times P\beta l / P\beta r$$

$$Rn = Rt \times Pnl / Pnr$$

where Rt is the ratio of cell density in the central areas of the left retina versus that of the right retina; Pαl, Pβl, and Pnl are the proportions of α, β and NAB cells in the left retina, respectively; and Pαr, Pβr, and Pnr are the proportions of α, β and NAB cells in the paired intact right retina, respectively.

Statistical analysis

Statistical analysis was performed by Student's *t*-test of unpaired parameters with PC software (StatView, SAS Institute, Cary, NC).

Table 1. Enhancing effect of intravitreally injected neurotrophic factors on total number of retinal ganglion cells surviving 2 weeks of optic nerve transection

Neurotrophic factors injected*[a]	Number of animals	Relative survival of total cells	
		%±SD	Increase[b]
Saline (control)	7	17±6.3	1
BDNF, 0.5 μg	4	27±6.0*	1.6
BDNF, 1.0 μg	3	29±3.3*	1.7
CNTF	3	31±8.1*	1.8
FGF2	4	11±3.7	0.6
GDNF	4	28±7.8*	1.6
NT-4	4	24±9.0	1.4
BDNF+CNTF	3	29±8.4*	1.7

* $P<0.05$ to control.

[a] Dose of each factor was 1.0 μg unless noted.

[b] Ratio of (surviving cell ratio in treated retina)/(surviving cell ratio in control retina).

RESULTS

Survival promoting effect of neurotrophic factors on axotomized RGCs

In the first series of experiments, we examined whether a single intravitreal injection of 1 μg or 0.5 μg BDNF, 1 μg CNTF, 1 μg GDNF, 1 μg FGF2, or 1 μg NT-4 enhances total numbers of surviving RGCs. We have previously reported that the ratios of RGC densities in the central area reflect well the ratios of total numbers of surviving RGCs after ON-transection versus those in the intact paired retinas (Watanabe et al., 2001). Accordingly, we measured the density of DiI-labeled RGCs in the central area of 540 μm × 360 μm in the ON-transected retina as compared with that in the intact paired retina, and estimated the ratios of total numbers of surviving RGCs.

Table 1 summarizes the ratios of densities of surviving RGCs in the central retinal area with an injection of one of the neurotrophic factors to those in the intact retina. On day 14 after ON transection, total RGC numbers in the retinas with saline injection (control) decreased to 17±6.3% ($N=7$) of the intact retinas. The value coincided with the reported value in the retina without injection, 17±8.5% (Watanabe et al., 2001). A single injection of BDNF, CNTF or GDNF resulted in an approximate 1.6–1.8-fold increase of surviving RGCs. In BDNF injections, the doses of 0.5 μg and 1.0 μg showed similar enhancement, suggesting that 0.5 μg was already a saturated concentration. A combination of 1.0 μg BDNF + 1.0 μg CNTF also enhanced the survival, but the effect was not additive to either BDNF or CNTF. This is consistent with the previous report in the rat retina, where no additive effect of BDNF + CNTF was obtained (Mey and Thanos, 1993). No enhancing effect on RGC survival was detected in the retinas with an injection of 1.0 μg FGF2 or 1.0 μg NT-4.

Enhancement of β cell survival by neurotrophic factors

To examine which type of cat RGCs is enhanced to survive by intravitreal injection of the neurotrophic factors, we esti-

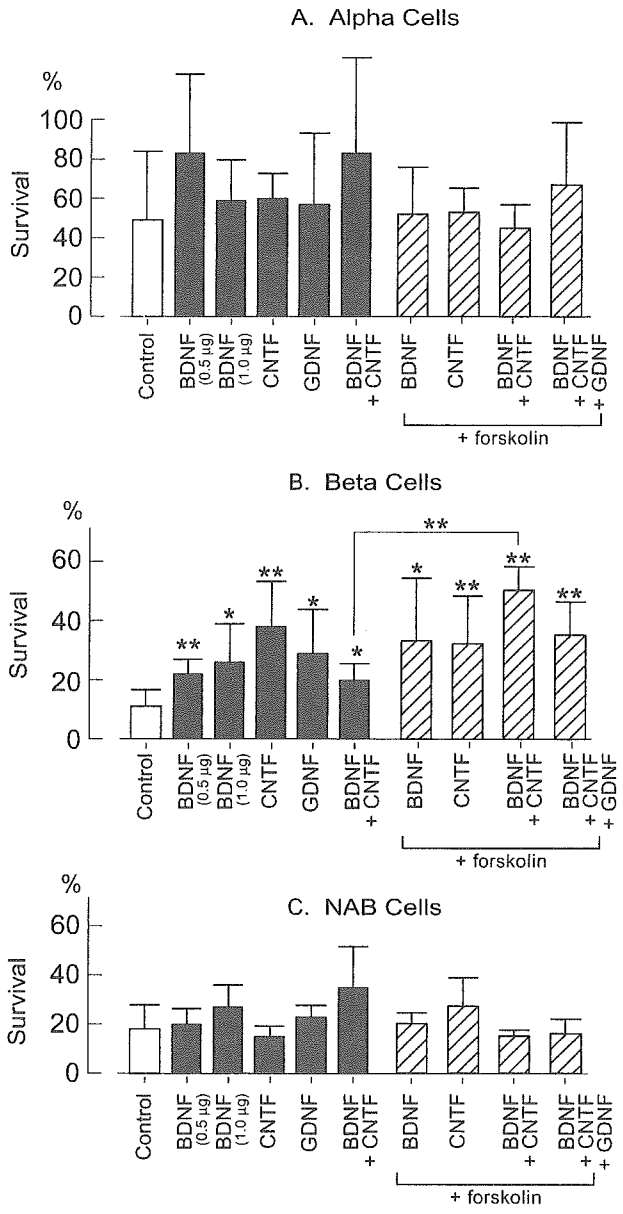

Fig. 1. Histograms of average with S.D. of survival ratios of α cells (1A), β cells (1B), and non-α/β (NAB) cells (1C). Dark bars illustrate survival ratios of retinal ganglion cells (RGCs) in the retinas with an injection of one or two neurotrophic factors, open ones saline (control). Hatched bars illustrate survival ratios of RGCs in the retinas with an injection of one neurotrophic factor or a combination of neurotrophic factors with forskolin. Asterisks on the bars indicate statistical significance against control, two asterisks on line in 2B between brain-derived neurotrophic factor (BDNF) + ciliary neurotrophic factor (CNTF) and BDNF + CNTF + forskolin. *, $P<0.05$, **, $P<0.01$.

mated the ratios of numbers of α, β or NAB cells in the left ON-transected retinas to those in the intact right retinas. Proportions of surviving α, β, and NAB cells in the paired retinas were obtained with systematic injections of LY into surviving RGCs, and then the ratios of every cell type were estimated as described above. As shown in Fig. 1, survival-enhancing effects of various neurotrophic factors were evident only in β cells. Numbers of surviving α cells in the retinas

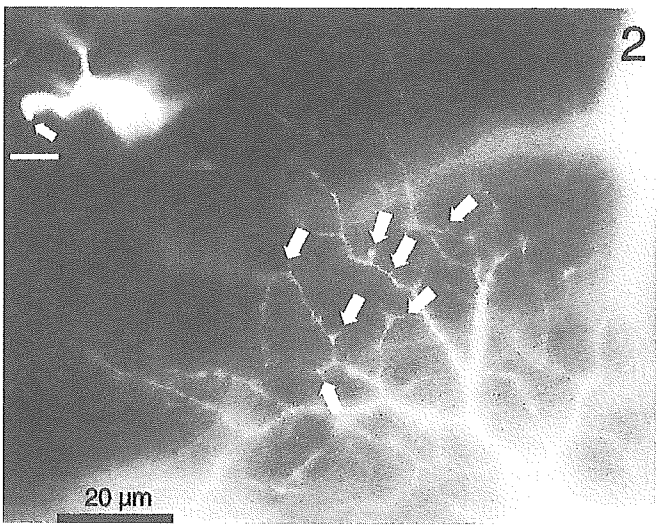

Fig. 2. Photomicrographs of dendrites of a Lucifer Yellow–injected β cell in the basic fibroblast growth factor-injected retina. Note many twiglike processes, some of which are indicated with arrows. A small process in the soma was recognized, possibly retracted axon (arrow, inset). Scale bars=20 μm. Contrast of the photographs was enhanced with Adobe Photoshop.

with the injections were not different from those in the control retinas. This is probably because α cells are the most resistant to axotomy (Watanabe et al., 2001); hence, their survival must be already maintained by some other mechanism. The ratios of numbers of surviving NAB cells did not increase in the retinas with neurotrophic factor injection, though it remains possible that survival of NAB cells may be enhanced at much higher concentrations of the neurotrophic factors.

The survival ratios of β cells increased in the retinas with an injection of BDNF, CNTF, or GDNF (Fig. 1B). These enhancing effects on β cell survival were similar in the retina with a single injection of either 0.5 μg or 1.0 μg of BDNF. In the retinas with an FGF2 injection, many twiglike processes were observed in dendrites of some β cells (Fig. 2). Because the morphological feature was so unusual and had no promoting effect on survival, we did not examine the effect of FGF2 in further experiments. Similarly, NT-4 did not promote the survival of β cells, so its effect was not examined further.

Effects of supplementation of forskolin on neurotrophic factors

It is reported that a high concentration of intracellular cAMP caused by forskolin enhances uptake and effect of neurotrophic factors in cultured RGCs (Meyer-Franke et al., 1995; Shen et al., 1999). We examined whether forskolin promotes the effects of BDNF, CNTF, and GDNF on the survival of axotomized RGCs *in vivo*. Injections of BDNF or CNTF with forskolin promoted numbers of surviving RGCs, but the effect was similar to that of an injection of BDNF or CNTF alone (not shown). On the other hand, a combined injection of BDNF + CNTF + forskolin (B+C+f) significantly enhanced the total numbers of surviving RGCs, 1.95-fold above that of the control. We then tested whether forskolin itself could promote

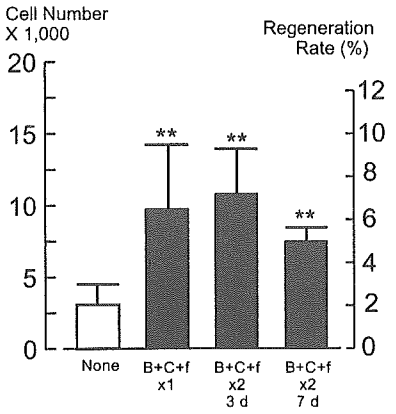

Fig. 3. Histogram of average with S.D. of total numbers of regenerated retinal ganglion cells (RGCs) with brain-derived neurotrophic factor + ciliary neurotrophic factor + forskolin (B+C+f) injections. Animal numbers were None, 8; B+C+f×1, 8; B+C+f×2 (3 day), 3; and B+C+f×2 (7 day), 3. The values in 'None' contain previous data (Watanabe et al., 1993). The left ordinate indicates total number of regenerated RGCs; the right, percentages against total RGC number as N=150,000. The values of all three experiments with B+C+f injection(s) were significantly higher than None at $P<0.01$. In the retinas with a second B+C+f injection on day 3 (×2, 3 day) or day 7 (×2, 7 day), values were not significantly different from those with one injection (×1).

survival of axotomized RGCs by injecting 0.1 mg forskolin and 1 μg NGF, which is not effective for the survival of central neurons. An injection of NGF + forskolin had no effect on the survival; the survival rate was similar to that of the control (16±4.1%). Therefore, forskolin itself did not enhance survival of axotomized RGCs.

Survival-promoting effect of one neurotrophic factor or a combination of neurotrophic factors and forskolin was again evident only in β cells (Fig. 1B). The ratio of β cells in injected retinas to that in the control was the greatest in the B+C+f–injected retinas, that is, 50% survival, 5.2-fold that of the control. The numbers of β cells in B+C+f–injected retinas were significantly higher than those in the retinas with BDNF + CNTF injection (2.43-fold). GDNF had no additive effect to the injection of B+C+f (0.70-fold to B+C+f). Given these results, we next examined the effect of intravitreal injection of B+C+f on axonal regeneration of RGCs.

Axonal regeneration with injections of B+C+f

The total number of regenerated RGCs was estimated in eight retinas with B+C+f injections and in eight retinas without the B+C+f injection (control). Fig. 3 compares the average number of regenerated RGCs. The data in control retinas include two values reported in our previous paper (Watanabe et al., 1993).

An intravitreal injection of B+C+f increased the average number of regenerated RGCs, 3.2-fold (10,379±5087, N=8) that of the average number in the controls (3252±458, N=8). The range of RGC numbers in B+C+f–injected retinas was from 5577 to 17,815, and these values were above the range in control retinas, from 1712 to 5040. To clarify whether additional injections of B+C+f further increase the number of regenerated RGCs,

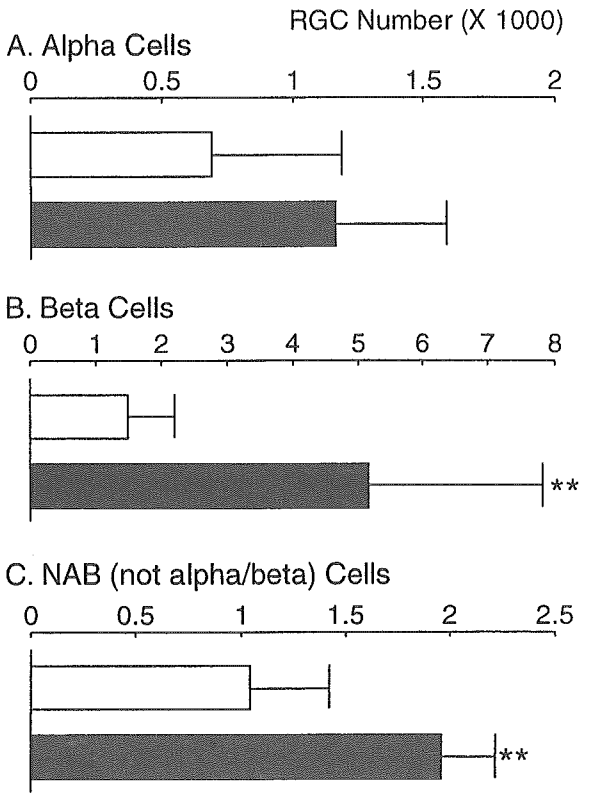

Fig. 4. Histograms of total numbers (average with S.D.) of regenerated α cells (A), β cells (B), and non-α/β (NAB) (C) cells with brain-derived neurotrophic factor + ciliary neurotrophic factor + forskolin (B+C+f) injections. Filled bars, B+C+f injections (N=6), blank bar, no injections (N=5). Because of no difference in total retinal ganglion cell numbers among one and two B+C+f injections (Fig. 3), cell numbers of B+C+f injections include values from retinas with one and two injections. The average number of regenerated β cells was 3.4-fold and that of NAB cells, 1.9-fold that of no injections, respectively. These values were significant at $P<0.01$.

we did a second B+C+f injection on day 3 or 7 (Fig. 3, right two bars). In the retinas with an additional injection of B+C+f on day 3, the number of regenerated RGCs was similar to those with single injections. In the retinas with an additional injection of B+C+f on day 7, the number was slightly lower than those in the retinas with single injections. The results suggest that there is no further promoting effect of B+C+f injections later than day 3.

The above experiments showed that a B+C+f injection was effective only for the survival of β cells. To examine whether a B+C+f injection also increases specifically the number of β cells in regenerated RGC populations, numbers of each type with regenerated axons were estimated from the total numbers of regenerated RGCs and the proportions of three cell types. Fig. 4 compares the average numbers of α, β, and NAB cells in the retinas with B+C+f injections and those in control retinas. The average number of β cells in the retina with B+C+f injection was 5167±2657, a 3.4-fold increase from that in retinas without B+C+f injection. The average number of NAB cells in the retinas receiving B+C+f injection increased by 1.9-fold and that of α cells by 1.7-fold of those in the retinas without the injection. The enhancement

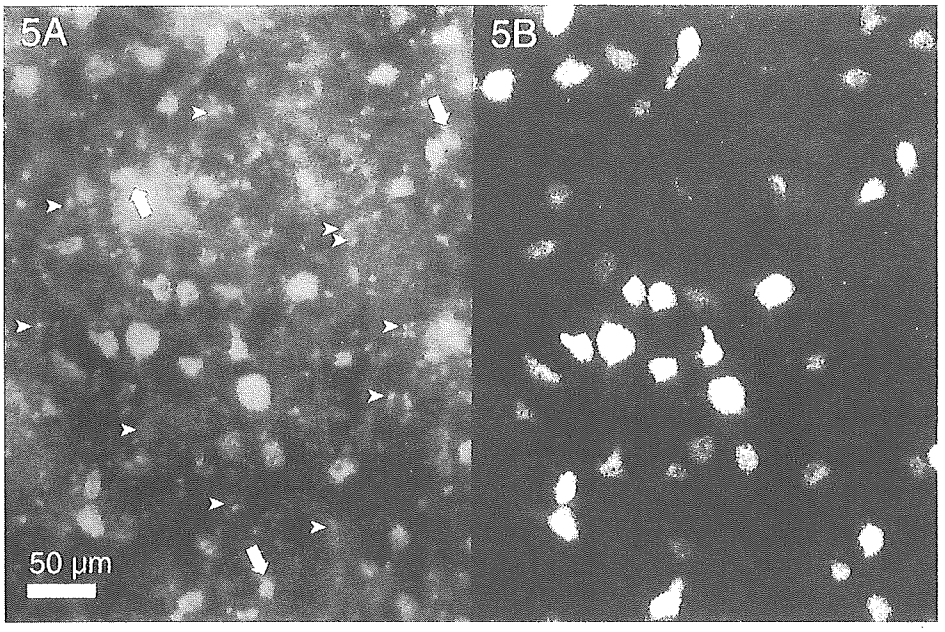

Fig. 5. Photomicrographs of surviving retinal ganglion cells (RGCs) (6A, DiI-labeled) and RGCs with regenerated axons (6B, fluorescein-labeled) in the same field. Those were regarded as labeled RGCs that had the soma larger than 8 μm with clear nuclear region. Debris, some of which is indicated with arrowheads, was not regarded as RGCs. Here 43 cells regenerated axons among 46 surviving cells. Arrows indicate RGCs without axonal regeneration. Contrast of the photographs was enhanced with Adobe Photoshop.

of B+C+f injection on axonal regeneration of the RGCs was greatest in β cells.

Does B+C+f directly facilitate axonal regeneration of RGCs?

The above experiments led to the notion that the B+C+f injection significantly enhances the survival of axotomized β cells and their axonal regeneration. However, it was unclear whether the B+C+f injection enhanced axonal elongation itself or only the survival. We then examined the ratios of RGCs with regenerated axons to those of total surviving RGCs, which was termed *'regeneration/survival ratio* (R/S ratio)', and how the B+C+f injection affected the ratios. Surviving RGCs in B+C+f–injected retinas were labeled with either of the following labeling methods: *in vitro* staining with Acridine Orange, which stains any living cells including displaced amacrine cells, and systematic central injections of DiI before the transplantation surgery. The DiI injections exclusively label RGCs (Watanabe et al., 2001). Regenerated RGCs were double-labeled by injections into the graft of dextran-fluorescein for DiI-labeled RGCs, or of dextran–Texas Red for Acridine-stained RGCs. Surviving RGCs in the retinas without B+C+f injections were labeled with Acridine Orange alone, whereas those in the B+C+f–injected retinas were labeled with Acridine Orange or DiI. Fig. 5 shows photomicrographs of surviving RGCs (6A, DiI-labeled) and regenerated RGCs (6B, fluorescein-labeled) in the same field. In this field, 43 cells regenerated their axons out of 46 surviving cells, and then the R/S ratio was 0.93.

When the surviving RGCs were stained with Acridine Orange, the average R/S ratio was 0.61±0.02 (N=3) in B+C+f injection, whereas the ratio was 0.68±0.01 (N=4) in the retinas without B+C+f injections (Fig. 6). There was no statistical difference between R/S ratios in the retinas with and without B+C+f injection. Because Acridine Orange may have labeled displaced amacrine cells, we measured R/S ratios for the surviving RGCs in B+C+f–injected retinas by DiI labeling as well. Then the average R/S ratio in B+C+f-injected retinas was 0.88±0.06 (N=9), being higher than those in Acridine Orange-stained retinas. There are two conceivable explanations for the discrepancy. First, Acridine Orange labeled all living cells in the ganglion cell layer so that the R/S ratio would be underestimated. Second, the systematic injections of DiI labeled most but not all RGCs, especially some NAB cells, which must have escaped from the DiI labeling (Watanabe et al., 2001); hence, the R/S ratio obtained by DiI labeling would be overestimated. Even taking these considerations into account, the results suggest that B+C+f injections did not directly increase the ability for axonal regeneration of surviving RGCs.

Most surviving β cells regenerated their axons along the PN graft

In the peripheral retina, a small but substantial number of DiI-labeled RGCs exhibited almost entire dendritic configurations. These cells survived axotomy for two months, but did not regenerate their axons along the PN graft. Systematic survey of these surviving and nonregenerated RGCs indicated that they were mostly of NAB

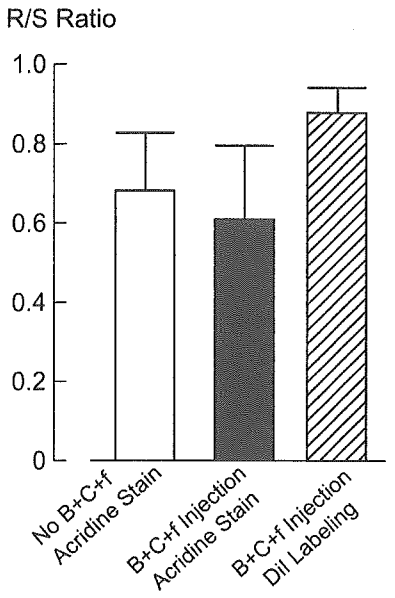

Fig. 6. Regeneration/survival (R/S) ratio of control retinas stained with Acridine Orange (blank bar, $N=4$), brain-derived neurotrophic factor + ciliary neurotrophic factor + forskolin (B+C+f)–injected retinas stained with Acridine Orange (filled bar, $N=3$), and B+C+f–injected retinas whose RGCs were labeled with DiI prior to the ON cut (hatched bar, $N=9$). There was no statistical difference between average values of the control and B+C+f with Acridine Orange staining.

cell type, but there were no clearly definable β cells in such populations of RGCs. This is consistent with the preceding observation that the survival and regeneration of β cells were significantly enhanced after B+C+f injections. In other words, it is suggested that a great majority of surviving β cells regenerated their axons when a permissive environment for axonal regeneration was provided with the PN graft.

DISCUSSION

Survival after ON transection is the first requisite for axonal regeneration of axotomized RGCs (Stichel and Müller, 1998; Goldberg and Barres, 2000). In the present study, we searched for new means to increase the number of surviving and regenerating RGCs in the adult cat retina. First, we found that single injections of B+C+f increased not only the number of RGCs surviving axotomy but also the number of regenerated RGCs. Furthermore, from cell type analysis we found that the effect of B+C+f injection was most prominent for the survival and axonal regeneration of β cells. This finding is very important because X-cells, the physiological counterpart of β cells, function to detect small visual objects, and their axonal regeneration is essential for functional recovery of acute vision.

Effect of BDNF, GDNF, and CNTF on survival of axotomized cat RGCs

Many papers have reported that BDNF, CNTF, FGF2, GDNF or NT-4 is effective in maintaining axotomized RGCs alive longer when injected into the vitreous (Stichel and Müller, 1998; Heiduschka and Thanos, 2000; Yip and So, 2000). In accordance with these reports, we demonstrated that an injection of BDNF, CNTF, or GDNF promoted the survival of axotomized cat RGCs, especially that of β cells, although NT-4, which shares the same potent receptor TrkB with BDNF, did not promote survival of cat RGCs (Peinado-Ramón et al., 1996). The present finding that axotomized cat RGCs survive better after intravitreal injections of BDNF is consistent with previous reports on promotion of survival of central neurons. We also found that an intravitreal injection of CNTF was effective for the survival of axotomized RGCs. This appears to be in conflict with previous studies on hamsters, which maintain that CNTF has no effect on survival of axotomized RGCs but promotes their axonal regeneration (Cho et al., 1999; Cui and Harvey, 2000). Because the rodent retina has very few RGCs corresponding to cat X cells (Fukuda et al., 1979; Hale et al., 1979; Huxlin and Goodchild, 1997) but some type II RGCs (Dreher et al., 1985) might correspond to β cells, survival enhancement by CNTF might not be detected in the rodent retina. On the other hand, CNTF is shown to promote survival of cultured rat RGCs (Meyer-Franke et al., 1995; Jo et al., 1999), as well as axotomized rat RGCs (Mey and Thanos, 1993). The effect of CNTF on survival of the RGCs may be indirect by factors secreted from activated Müller cells. The expression level of glial fibrillar acidic protein in Müller cells is enhanced by CNTF (Wen et al., 1995; Peterson et al., 2000), and the activated Müller cells secrete some trophic factors suitable for survival of RGCs (Chun et al., 2000; Wahlin et al., 2000).

GDNF has been shown to protect cell death of axotomized dopaminergic neurons (Beck et al., 1995; Yurek, 1998), motor neurons (Oppenheim et al., 1995; Yan et al., 1995), and axotomized rat RGCs (Koeberle and Ball, 1998; Yan et al., 1999). We also showed that the effect of GDNF on the survival of axotomized RGCs was as potent as that of BDNF or CNTF. Because the effect of GDNF was not additive to CNTF in B+C+f injection, GDNF may also activate Müller cells to secrete trophic factors. However, the mechanism underlying the survival-promoting effect of GDNF on axotomized RGCs has not yet been closely examined.

Effects of adding forskolin to neurotrophic factors on survival

Improvement of survival of cultured RGCs has been reported by adding forskolin to the medium containing BDNF (Meyer-Franke et al., 1995). Meyer-Franke et al. (1998) have suggested that depolarization and elevation of intracellular cAMP level rapidly recruit TrkB to the plasma membrane by translocation from intracellular stores and that as a consequence RGCs survived better than in a single application of BDNF.

In the present *in vivo* study on the cat RGCs, however, addition of forskolin to the vitreous only slightly enhanced the effect of BDNF on the survival of axoto-

mized RGCs. It is not clear why the effect of BDNF + forskolin was insignificant *in vivo*, but intravitreally injected BDNF molecules may have been quickly internalized into RGCs via TrkB receptors recruited by cAMP. In the case of CNTF + forskolin injections, there was no additive effect of forskolin, either. This suggests that CNTF receptors may not be recruited with cAMP in RGCs. In this context, it is very difficult to understand why B+C+f injection revealed a much greater enhancing effect on survival of RGCs than BDNF or CNTF alone or by adding forskolin to each. When GDNF was added to the B+C+f, it did not show an additive effect to the B+C+f. This implies that CNTF and GDNF may similarly activate Müller cells, and their receptors may not be affected with cAMP.

Enhancement of axonal regeneration with B+C+f injection

We found that the B+C+f injections also enhanced axonal regenerations of cat RGCs, and the enhancement was especially conspicuous in β cells. BDNF has been reported to be a specific molecule for survival of axotomized RGCs (Mey and Thanos, 1993; Peinado-Ramón et al., 1996; Chen and Weber, 2001), but also stimulates their axonal branching in the retina (Mansour-Robaey et al., 1994; Sawai et al., 1996). However, it has been reported that BDNF does not increase the number of regenerated RGCs by PN transplantation (Mansour-Robaey et al., 1994). No increase of regenerated RGCs *in vivo* can be understood by the fact that once axons retract in the retina they cannot enter the ON again (Sawai et al., 1996) because the optic disc contains repulsive molecules such as tenascin (Bartsch et al., 1994) and collapsin (Luo et al., 1995). BDNF seems to be insufficient for intraretinally regenerated axons to overcome the barrier molecules around the optic disc. As a result, BDNF injection may not facilitate *in vivo* axonal regeneration along the PN graft.

In the present study we obtained a 3.2-fold increase of the regenerated RGCs after intravitreal B+C+f injections, which contained CNTF and forskolin besides BDNF. Because CNTF facilitates axonal regeneration (Cui and Harvey, 1995, 2000; Jo et al., 1999), combination of CNTF and BDNF may effectively function to keep more RGCs alive before axonal retraction. Thus, the combination of BDNF and CNTF may facilitate axonal regeneration along the PN graft.

It is more probable that cAMP facilitates axonal regeneration of RGCs. The facilitation of axonal regeneration by cAMP has been pointed out from the fact that cAMP concentration is much higher in young neurons (Cai et al., 2001; DeBellard et al., 1996). In young animals axonal regeneration is enhanced by myelin-associated glycoprotein (MAG), which is a potent inhibitor for axonal sprouting of adult neurons (DeBellard et al., 1996). Cultured Xenopus neurons with a high cAMP concentration show blockage of inhibition of axonal sprouting on MAG (Song et al., 1998). When the cAMP level is elevated in cultured RGCs of P5 rats with permeable cAMP homologue, the RGCs extend sprouts on MAG much longer than RGCs with normal cAMP concentrations (Cai et al., 1999, 2001). In the present experiments, the cAMP level must have been elevated in the RGCs so that they could easily extend their axons across barriers at the junction. In fact, survival of NAB cells was not enhanced by the B+C+f injection, but the average number of regenerated NAB cells increased 1.9-fold. In conclusion, high cAMP may facilitate *in vivo* axonal regeneration of adult RGCs.

Why the B+C+f injection is specifically effective for β cells

We found that B+C+f specifically enhances the survival of β cells. Both their survival and axonal regeneration were significantly enhanced (by 4.7- and 3.4-fold, respectively). In contrast, the survival of a or NAB cells was not enhanced substantially by B+C+f, although a 1.9-fold elevation of axonal regeneration was noted in NAB cells (Fig. 4). Why was there such a clear difference among the types of cat RGCs? We observed that in the peripheral retina, most surviving RGCs without axonal regeneration were of the NAB type and all the surviving β cells regenerated their axons along the PN graft. It has been shown that axotomized RGCs are unable to extend their axons into the ON once they retract their axons from the optic disc (Sawai et al., 1996). Therefore, it is postulated that axotomized β cells with retracted axons may have degenerated, whereas NAB cells may have survived without axonal regeneration. If this is actually the case, some intracellular death mechanism might be switched on in β cells in response to axotomy and encounters with repulsive molecules at the optic disk. On the other hand, such a mechanism might be shut down by activation of TrkB and related signaling pathways after B+C+f applications. The notion is consistent with our previous observation on the survival time course of axotomized cat RGCs that β cells specifically suffer from early phases of cell death shortly after ON transection (Watanabe et al., 2001).

The present results imply that the expression of TrkB receptors facilitates survival of β cells by shutting down the pathway to cell death, which may be induced by axotomy and/or repulsive molecules. The receptors possibly affect axonal regeneration of β cells when intracellular cAMP is raised. To clarify intracellular mechanisms for β cell death and axonal regeneration, detailed mechanisms underlying these processes should be explored by using *in vitro* preparations of isolated β cells in comparison with other cell types of cat RGCs.

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48. 正常および網膜変性モデルラットへの光ストレスの影響

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(弘前大)

研究要旨　網膜色素変性症は遺伝性網膜変性疾患であり、治療法が確立されていない難病である[1]。その研究モデルとして用いられる網膜光傷害は網膜変性に至る分子病態を含めて不明な点が多い[2,3]。今回、我々は網膜光傷害および遺伝性網膜変性疾患の分子病態を詳しく解析する目的で、正常ラット及び網膜変性ラットに対し様々な条件の光ストレスを与えて系統的な検討を行った。その結果、網膜光傷害と網膜変性ラットにおいてロドプシンのリン酸化状態が過度に継続するという分子病態の共通性が確認された。今回の結果は、動物種は異なるがヒト網膜色素変性症を含む網膜変性疾患に対して過度な光ストレスを与えることは有害である可能性を示唆するものと思われるが、この点についてはさらに今後の検討が必要であると思われる

A. 研究目的

本研究では正常ラットおよび遺伝性網膜変性モデル動物である The Royal College of Surgeons (RCS)ラットへの光ストレスの影響を系統的に検討することを目的とした。

B. 研究方法

RCS と正常ラット (Sprague Dawley rat, Brown Norway rat) に対して様々な照度・照射時間で光照射を行ない、光学顕微鏡による組織学的検討、網膜電図 (ERG) による機能評価、ロドプシンの再生、ロドプシンの脱リン酸化速度の検討[4]、細胞内 cGMP 濃度の測定、TaqMan®PCR を用いた神経栄養因子の網膜内発現変化を検討した。

(倫理面への配慮)

本研究における動物を用いた実験は、弘前大学付属動物実験施設のガイドラインをふまえ、学内委員会の許可を得て行った。

C. 研究結果

種々の条件(650, 1300, 2500 or 5000 lux , 24 hrs)で RCS および正常ラットに光照射したところ、照度依存的に ERG 振幅が低下した。組織学的変化として光照射から1週間で照度依存的に外顆粒層の菲薄化がみられた。RCS ラットにおいても照度依存的に ERG 振幅低下と外顆粒層の菲薄化がみられた。光ストレス後のロドプシンの再生には RCS および正常ラットともに有意差はみられなかった。RCS および正常ラットともに光ストレスを与えた群では有意にロドプシンの脱リン酸化時間が延長していた。また、RCS ラットでは、もともとロドプシンの脱リン酸化時間が延長していることがわかった。RCS ラットおよび光ストレスを与えた SD ラットでは光依存的な網膜内 cGMP 濃度

低下が十分におこらなかった。RCS および正常ラットともに各種神経栄養因子の網膜におけるmRNA レベルでの発現に有意な変化はみられなかった。

D. 考察

ロドプシンが異常に長くリン酸化状態にあることが、遺伝子異常による視細胞変性と網膜光傷害の共通機序と考えられた。

この機序により、網膜内でどの様な病態に陥るかを推測すると、以下の状態が考えられた。

1）ロドプシンのリン酸化状態の亢進により光依存性の transducin の活性が抑制され、細胞内に cGMP が蓄積する。2）細胞内の cGMP 濃度の低下が起こらないため cGMP gated channel が開いた状態が続く。3）cGMP gated channel から細胞内への Ca^{2+} 流入が続き、細胞内 Ca^{2+} 濃度が上昇する。4）Ca^{2+} 依存的なアポトーシス経路が活性化する。

E. 結論

今回の検討から以下を結論とする。

光ストレスはロドプシンの脱リン酸化を著明に延長させるため、光情報伝達経路の異常を惹き起こしはするが、光ストレスによる網膜保護効果はみられなかった。今回の研究から、動物種は異なるがヒト網膜色素変性症を含む網膜変性疾患に対して過度な光ストレスを与えることは有害である可能性を示唆するものと思われるが、この点についてはさらに今後の検討が必要であると思われる。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

なし

2. 学会発表

なし

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

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49. 網膜虚血再潅流障害に対するD-アロースの神経保護効果の機序

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([1)]香川大、[2)]香川大脳神経生物学、[3)]細胞情報生理学)

研究要旨　網膜虚血再潅流障害に対して希少糖（自然界に微量にしか存在しない単糖）の一つであるD-アロースに神経保護効果を有することを報告したが、その作用機序およびD-アロースの眼内移行濃度についてラット虚血再潅流モデルを用いて検討した。腹腔内にD-アロースとグルコースを虚血開始30分前に投与した。1週間後に眼球を摘出し、網膜組織障害を検討したところ、D-アロースとグルコースを同時に注入することによりD-アロースの神経保護効果は阻害された。またD-[1-^{14}C]アロース液（100 mg/1.11 MBq/2.0 ml/kg）を5 ml/kg(555 Bq/μl)の用量で腹腔内に投与し、その30分後に液体シンチレーションスペクトロメーターを用いて放射能を測定した。D-[1-^{14}C]アロースの血液中放射能濃度は 42.8 Bq/μl、硝子体中放射能濃度は46.4 Bq/μl でそれぞれ腹腔内投与量の7.7%、8.4%であった。D-アロースはグルコースと競合的に働くことにより神経保護効果を有する可能性が示唆され、また投与量の約８％のD-アロースが眼内に達した。

A．研究目的

昨年及び一昨年の本会において網膜虚血再潅流障害に対して希少糖（自然界に微量にしか存在しない単糖）の一つであるD-アロースに神経保護効果を有することを報告した。今回その作用機序およびD-アロースの眼内移行濃度を検討したので報告する。

B．研究方法

ラットの前房内圧を130 mmHgに上昇させることにより網膜虚血を行い、45分間虚血を行った。腹腔内に200 mg/kg D-アロースと200 mg/kg グルコースを虚血開始30分前に投与した。1 週間後に眼球を摘出し、網膜切片を作成しH-E 染色を行うことにより網膜組織障害を検討した。またD-[1-^{14}C]アロース液(100 mg/1.11 MBq/2.0 ml PBS/kg)を10 ml 調製し、これを投与液とした。555 Bq/μl の用量を腹腔内に投与しその30分後に血液は尾静脈から採取し、硝子体は眼球を摘出した後採取した。放射能は液体シンチレーションスペクトロメーターを用いて測定した。

C．研究結果

D-アロースとグルコースを同時に注入することによりD-アロースの神経保護効果は阻害された。
また555 Bq/μl のD-[1-^{14}C]アロースを腹腔内投与30分後の血液中放射能濃度は42.8 Bq/μl、硝子体中放射能濃度は 46.4 Bq/μl で腹腔内投与量の 7.7%、8.4%であった。

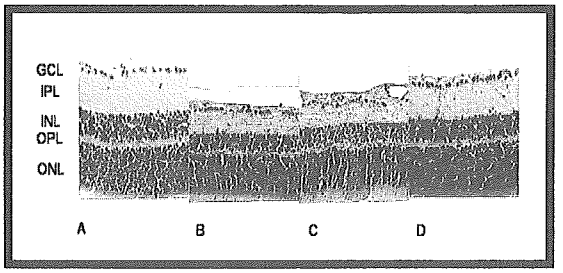

図１　グルコース同時投与の影響
A:コントロール、B:虚血-生食、C:虚血-D-アロース+グルコース、D:虚血-D-アロース
GCL:神経節細胞層、IPL:内網状層、INL:内顆粒層、OPL:外網状層、ONL: 外顆粒層

表 硝子体と血液中でのD-アロースの分布

硝子体	46.4±5.2	(8.4 ± 1.0)
血液	42.8±3.0	(7.7 ± 0.5)

D．考察

グルコースとD-アロースを同時に投与することによりD-アロースの神経保護効果が抑制された。D-アロースがグルコースの膜輸送を阻害する可能性があることが報告されており[1]、今回の結果からもD-アロースとグルコースが競合的に働くことにより神経保護効果を有する可能性が示唆された。

E．結論

D-アロースはグルコースと競合的に働くことにより神経保護効果を発揮する可能性が示唆された。また、D-アロースを腹腔内に投与した場合、血液中とほぼ同程度で腹腔内投与量の約８％のD-アロースが眼内に達した。

F．健康危険情報

なし

G．研究発表

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２．学会発表

なし

H．知的財産権の出願・登録状況

１．特許取得

2003 年 5 月 22 日に希少糖の生理活性作用の利用方法および希少糖を配合した組成物（生理番号：PCT-03-RS01）の名称で特許の出願を行った。

２．実用新案登録

なし

３．その他

なし

I．参考文献

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50. イソプロピル ウノプロストン点眼液の網膜保護効果についての検討

伊藤　忠、大黒　浩、大黒幾代、間宮和久、石川　太
目時友美、山崎仁志、横井由美子、中澤　満
(弘前大)

研究要旨　目的) イソプロピル　ウノプロストン（以下ウノプロストン）は眼圧下降効果に加えて、眼組織血流量の増加による神経保護効果を有する抗緑内障点眼薬である。この神経保護作用は緑内障以外の網膜細胞にアポトーシスをおこす網膜変性疾患にも有効である可能性がある。今回我々は網膜変性モデルラットにウノプロストンを連日投与して、機能的、形態学的及びアポトーシス関連遺伝子の変化について検討した。　方法) 日齢21日のRCSラットとP23Hラット（ヘテロ）にウノプロストンを１日２回、14日間連日点眼投与を行った。コントロールとして基剤の投与も同様に行った。その後、ERGを用いた機能的評価、眼球摘出後の組織学的評価及び網膜採取後のTaqMan®PCRを用いてアポトーシス関連遺伝子の変化について検討した。　結果) RCSラットではいずれの評価においてもウノプロストン投与群と基剤投与群との間で明らかな差は認めなかった。P23HラットではERGにおけるｂ波及び組織における ONL 層の厚さが基剤投与群と比べてウノプロストン投与群で有意に保たれていた。TaqMan®PCR では両群で明らかな差はみられなかった。　考察) ウノプロストン投与により、ある種の網膜変性モデル動物でも機能的及び組織学的変化の抑制がみられ、その有効性が示唆された。今後さらに長期投与における変化の検討を要すると考えられる。

A．研究目的

ウノプロストンは房水流出促進による眼圧下降効果に加えて、眼組織血流量の増加[1-5]による神経保護効果を有する抗緑内障点眼薬である。後者の神経保護作用は緑内障以外の網膜細胞にアポトーシスをおこす網膜変性疾患にも有効である可能性を示唆している。今回我々は網膜変性モデルラットにウノプロストンを連日点眼投与して、その機能的、形態学的およびアポトーシス遺伝子発現の変化について検討した。

B．研究方法

網膜変性モデル動物であるRCSラットとロドプシン遺伝子 P23H トランスジェニックラット(ヘテロ)に、日齢21日よりウノプロストンを１日２回、２週間連続点眼投与を行った。コントロール群には基剤投与を同様に行った。機能的評価では点眼投与が終了した時点で24時間暗順応を行い、網膜電図(ERG)を用いてａ波およびｂ波の振幅を計測した。組織学的評価では眼球摘出後に網膜断層の標本を作製し、各層を比較した。アポトーシス遺伝子発現の変化については、

網膜採取後アポトーシス関連因子であるcaspase3、caspase8、caspase9、bcl-2、bcl-x、Baxの発現の変化をTaqMan®PCRを用いて転写レベルで検討した。

C．研究結果

ERGでは、RCSラットにおいてa波、b波ともにウノプロストン投与群と基剤投与群で明らかな差はみられなかった。P23Hラットでは、a波の振幅は両群で差はみられなかったが、b波の振幅はウノプロストン投与群で有意に保たれていた。組織像では、RCSラットでは両群で各層の厚さに差はみられなかった。P23Hラットではウノプロストン投与群の外顆粒層が基剤投与群のそれと比較して優位に保たれていた。アポトーシス関連因子については、いずれのラットにおいても両群間で明らかな差はみられなかった。

D．考察

P23Hラットでは、ウノプロストン投与群で機能的、組織学的変化の抑制がみられた。このことは、ウノプロストンの薬効によって網膜変性が抑制されている可能性があると考えられた。しかし、caspase等のアポトーシス関連遺伝子の発現に影響はみられなかった。今回の実験では投薬期間が2週間と短期間だったので、今後は長期投与における変化の評価や、分子レベルでの変化の検討も要すると考えられた。

E．結論

ウノプロストン投与により、ある種の網膜変性モデル動物でも機能的、組織学的変化の抑制がみられ、その有効性が示唆された。今後さらに長期投与における分子レベルでの評価も含めた検討を要すると考えられた。

F．健康危険情報

なし

G．研究発表

1．論文発表

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2．学会発表

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H．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

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動脈血流速度に及ぼす影響．あたらしい眼科 15(2)：281-284，1998

51. Rho/ROCK 阻害剤によるネコ網膜神経節細胞の軸索再生

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研究要旨　成ネコを用いて、培養網膜の突起伸展と、挫滅された網膜神経節細胞（RGCs）の軸索再生を、新規 Rho/ROCK 阻害剤の Y39983 が促進するかどうかを調べた。網膜切片を Y39983 を含む培養液で培養し、固定後抗 TUJ1 抗体で神経突起を免疫染色した。視神経挫滅は 6-0 糸に 20 g の張力を 1 分間適用した。挫滅直後に Y39983 を硝子体と挫滅部に注入した。12 日後、WGA-HRP を硝子体に注入し 2 日後に固定した。視神経の凍結切片を作成し、TMB 反応で順行性標識された軸索を可視化した。

網膜培養において Y39983 の神経突起伸展促進作用は、網膜中心部から辺縁部までのすべての部位の RGCs において、3 および 10 μM で最大の突起伸展作用が得られた。20μM 以上では効果は減弱し、30μM 以上になると神経突起伸展を完全に阻害した。また、挫滅された RGCs 軸索は、挫滅部位を越えて視神経内の中枢側へ再生していた。網膜培養と同様に、10 μM で再生促進効果が最大であった。視神経挫滅から 1 週後に Y39983 を硝子体に再度注射すると、再生軸索数がさらに増加した。

A. 研究目的

中枢神経の軸索は、障害されたら再生しない。しかし近年、中枢神経のネットワーク障害に対する研究は飛躍的に進歩し、軸索再生阻害の分子メカニズムは次第に明確になりつつある。すなわち、軸索伸展抑制分子(Nogo, MAG, OMgp など)が、受容体を介して神経細胞内の Rho/ROCK 経路を活性化し、軸索進展を阻害する。したがって Rho あるいは ROCK の活性を抑制することで、中枢神経の軸索の再生促進が期待される。新規 Rho/ROCK 阻害剤、Y39983 は、ラット網膜神経節細胞（RGC）の移植末梢神経への、軸索再生を促進する（Takayama et al., 2004)。今回我々は、成ネコを用いて、培養網膜の突起伸展と、挫滅された RGCs 軸索の再生を観察し、これらを Y39983 が促進するかどうかを調べた。

B. 研究方法

網膜培養：笑気とハロセンの麻酔下の成ネコから、眼球を摘出し、網膜を無菌的に遊離した。1 辺約 0.5 mm の小片とし、0℃の低温下でコラーゲン混合液中に浸した。37℃に急速加温してコラーゲンをゲル化させ、組織片を包埋した。

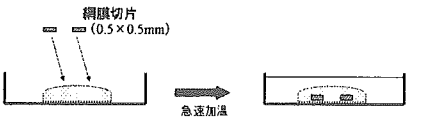

図 1　網膜培養法

ゲル化したコラーゲン中に包埋することにより、網膜組織片は、コラーゲンゲル中に非常に安定した状態で保持されることになり、網膜の神経細胞、グリア系の細胞などをすべて含んだ状態で培養するため、よ

り生体の眼球内に近い状態で神経再生を観察できる。網膜細片を Y39983 を含む DMEM で 14 日間培養し、3% paraformaldehyde in PBS で固定した。伸長した神経突起を抗 TUJ1 抗体で免疫染色し、TUJ1 陽性の突起の数および最長の長さを測定した。ネコ網膜における RGC の分布図に従って、細胞密度の一致した部位（central area、intermediate area、peripheral area）より網膜小片を採取し、各部位間の神経突起数の違いも検討した。

視神経挫滅：麻酔下の成ネコの前頭洞および眼窩上壁を開放後、左視神経を露出した。左視神経に巻いた 6-0 ナイロン糸に 20 g（0.2 N）の張力を 1 分間適用し、視神経を挫滅した。挫滅直後に 30G 注射針で Y39983 を、硝子体および挫滅部へ注入した。対照眼には PBS を同様に注射した。12 日後、WGA-HRP 0.5mg を硝子体に注入して RGCs の軸索を順行性に標識し、2 日後に固定液（1% paraformaldehyde + 1% glutaraldehyde in PBS）で潅流固定した。視神経を摘出してゼラチン包埋し、30 μm の凍結切片を作成した。切片を TMB 反応で処理し、順行性に標識された軸索を可視化した。挫滅部を超えて 0.5mm 以上伸長した軸索数および、最長の軸索長を測定した。

（倫理面への配慮）

今回の実験は、発達障害研究所動物実験委員会の承認を得た後、ARVO の動物実験規定に従って行われた。実験動物の苦痛は最小限となるように配慮がなされた。

C．研究結果

網膜培養：対照培養では網膜から伸長した再生突起はごくわずか認めるのみであったが、Y39983 を添加すると、伸長した再生突起がコラーゲンゲル中を 3 次元的に伸長しているのが観察された。これらの突起の先端に成長円錐（growth cone）が認められた。TUJ1 陽性の神経突起数を検討したところ、Y39983 による神経突起伸展促進作用は、網膜中心部から辺縁部までのすべての部位において、3～10 μM で最大であった。網膜の部位による最大の突起伸展作用の Y39983 濃度に差はみられなかった。Y39983 濃度 20 μM 以上では突起伸展作用は減弱し、30 μM 以上になるとむしろ突起伸展を阻害した。伸展した突起の最長の長さも、同様に 3～10 μM で最長のものが観察され、20 μM 以上では効果は減弱した。最長突起の長さも、網膜の部位別での差は認められなかった。

視神経挫滅：対照動物の視神経では、挫滅部を超えて再生する軸索はほとんど認められなかった。一方 Y39983 を眼球内と挫滅部に注入した視神経では、挫滅された軸索が挫滅部位を越えて、視神経中枢部位へ再生しているのが観察された。軸索伸展作用は、網膜培養と同様に 10 μM で再生軸索数が最大であった。また視神経挫滅から 1 週後に、Y39983 を硝子体に再度注射すると、挫滅直後のみの単回投与に比べて、再生軸索数が増加した。100 μM 注入の視神経でも再生軸索がみられた。伸展軸索の最長の長さも同様に、10 μM で最も長かった。視神経挫滅直後のみの単回投与と 1 週後に再注射した 2 回投与では、再生軸索の最長の長さに有意差は見られなかった。

D．考察

1. 神経突起伸展促進作用を示す濃度は、網

膜中心部から辺縁部までの各部位の神経節細胞において差は認めなかった。

2. In vivo での実験では 100μM の濃度でも再生軸索がみられたが、これは Y39983 の代謝により実際の作用濃度が薄まったためであると考えられる。

3. Y39983 を２回注入した視神経で再生線維が増加していたことから、挫滅後１週後でも視神経線維が新たに再生した可能性が示唆される。

4. Rho/ROCK pathway 以外への作用の有無、他剤との比較、副作用等については現在検討中である。

E. 結論

新規 Rho/ROCK 阻害剤 Y39983 は、培養下および軸索を挫滅されたネコ網膜神経節細胞の、軸索再生を促進する。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

なし

2. 学会発表

なし

H. 知的財産の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

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