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Upregulation of IGF-I in the goldfish retinal ganglion cells during the early stage of optic nerve regeneration

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**Running title**: IGF-I in goldfish optic nerve regeneration

**Abstract**

Goldfish retinal ganglion cells (RGCs) can regrow their axons after optic nerve injury. However, the reason why goldfish RGCs can regenerate after nerve injury is largely unknown at the molecular level. To investigate regenerative properties of goldfish RGCs, we divided the RGC regeneration process into 2 components: 1) RGC survival, and 2) axonal elongation processes. To characterize the RGC survival signaling pathway after optic nerve injury, we investigated cell survival/death signals such as Bcl-2 family members in the goldfish retina. Amounts of phospho-Akt (p-Akt) and phospho-Bad (p-Bad) in the goldfish retina rapidly increased 4-5 folds at the protein level by 3-5 days after nerve injury. Subsequently, Bcl-2 levels increased 1.7 folds, accompanied by a slight reduction in caspase-3 activity 10-20 days after injury. Furthermore, level of insulin-like growth factor-I (IGF-I), which activates the phosphatidyl inositol-3-kinase (PI3K)/Akt system, increased 2-3 days earlier than that of p-Akt in the goldfish retina. The cellular localization of these molecular changes was limited to RGCs. IGF-I treatment significantly induced phosphorylation of Akt, and strikingly induced neurite outgrowth in the goldfish retina *in vitro*. On the contrary, addition of the PI3K inhibitor wortmannin, and IGF-I antibody

inhibited Akt phosphorylation and neurite outgrowth in an explant culture. Thus, we demonstrated, for the first time, the signal cascade for early upregulation of IGF-I, leading to RGC survival and axonal regeneration in adult goldfish retinas through PI3K/Akt system after optic nerve injury. The present data strongly indicate that IGF-I is one of the most important molecules for controlling regeneration of RGCs after optic nerve injury.

*Keywords: goldfish, retina, retinal ganglion cells ,IGF-I, p-Akt, survival, optic nerve regeneration*

*Abbreviations*: GCL, ganglion cell layer; IGF-I, insulin-like growth factor; INL, inner nuclear layer; ON, optic nerve; PI3K, phosphatidyl inositol-3-kinase; RGC, retinal ganglion cell

## 1. Introduction

Axonal injury of adult mammalian CNS neurons generally leads to retrograde neuronal degeneration and cell death. In parallel paper, we reported caspase-3 dependent apoptosis and downreguration of insulin-like growth factor-I in rat retinal ganglion cells (RGCs) after optic nerve (ON) injury. On the other hand, RGCs of lower vertebrates such as fish can successfully regenerate their axons after ON injury (Arora and Sperry, 1957). There have been many studies on morphological and behavioral changes involved in fish ON regeneration (Grafstein 1975; Springer and Agranoff , 1977; Benowitz et al., 1981; Kato et al., 1999, 2006; Rodger et al., 2005 ). Furthermore, biochemical and genetic approaches for identifying molecules involved in ON regeneration in fish have recently accumulated (Schwalb et al., 1996; Liu et al., 2002; Matsukawa et al., 2004a; Fisher et al., 2004; Sugitani et al., 2006). However, there are few reports clarifying the reason why fish can regenerate optic axons after injury. In the present study, we examined goldfish RGCs as counterparts of rat RGCs with respect to regenerative properties after ON injury. The process of goldfish ON regeneration might be divided into 2 mechanisms: 1) RGC survival and 2) axonal elongation. We first measured enzymatic activities and protein levels of cell

survival/death-related molecules such as members of the Bcl-2 family, which are major pro-apoptotic and anti-apoptotic proteins or the phosphatidyl inositol-3-kinase (PI3K)/Akt system which plays a central role in cell survival (Yamaguchi and Wang, 2001). The present study clearly showed early upregulation of phospho-Akt (p-Akt) and subsequent upregulation of Bcl-2 activity with a concomitant downregulation of caspase-3 activity in the goldfish RGCs after ON injury. Furthermore, we observed an earlier upregulation of insulin-like growth factor-I (IGF-I) than that of p-Akt in the goldfish RGCs after nerve injury. Addition of IGF-I to the culture medium dose- and wortmannin-dependently induced activation of Akt and axonal elongation in adult goldfish RGCs. Thus, the present data demonstrated, for the first time, a signaling pathway for the initial upregulation of IGF-I leading to optic nerve regeneration via the PI3K/Akt system in the goldfish RGCs, after nerve injury.

## 2. Experimental procedures

### *2.1. Animals and surgery*

The common goldfish (*Carrassius auratus;* length, 6-7 cm tip-to-tip) was used throughout this study. The experiments were carried out in accordance with Committee on Animal Experimentation of the Kanazawa University. Goldfish were anesthetized in ice-cold water. The ONs were crushed using tweezers 1 mm away from the eyeball for 10 s, to carefully avoid vessels. After this treatment, goldfish were kept in appropriate conditions until the indicated time points after ON crush.

### *2.2. Cell counts of RGCs*

To evaluate survival of goldfish RGCs after ON crush, we used a retrograde neurotracer, fluorogold. The ON was treated with small particles of fluorogold (10 mg/ml) at the crushed site for 1-2 days. The number of RGCs in the flat-mounted goldfish retina was counted with a fluorescent microscope. More than 3 retinal preparations were analyzed for each sample at indicated time points after ON crush. Values were expressed as means ±

S.E.M. of RGCs before and after ON crush (flat-mounted retinas).

*2.3.Caspase-3 activity in the retina*

Retinal samples at indicated time points following ON injury were homogenized with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM dithiothreitol, and then centrifuged at 1,800g at 4 ˚C for 1 h. The assay method was the same as that in parallel paper (Homma et al., 2007).

*2.4. Western blotting of cell survival and death signal molecules*

Goldfish retinal extracts were prepared at the indicated times after ON injury. Retinas were immersed in Tris-HCl buffer (pH 7.4) containing a protease inhibitor cocktail (Sigma), and were then sonicated and centrifuged at 1,000g for 10 min , as described previously (Koriyama et al., 2003). The electrophoresis and blotting were the same as that in parallel paper (Homma et al., 2007).

*2.5. Immunohistochemistry*

Fixation and cryosectioning of retinal samples were carried out as described previously (Devadas et al., 2001). The immunohistochemical staining for retina was the same as that in a parallel paper (Homma et al., 2007).

*2.6. RNA isolation and RT-PCR*

Total RNA was isolated from retinas at appropriate times after ON injury using Sepasol(R)-RNA Ⅰ (Nacalai tesque, Japan). RT-PCR was performed using the RNA PCR kit (AMV) Ver.3 (Takara, Japan) including the AMV reverse transcriptase XL, and was performed 3 times with isolated RNA (500 ng). Primer sequences (Forward: 5′-TGTACCATGCGCTGTCTCTC-3′, Reverse: 5′-GGCTCGAGTTCTTCTGATGG-3′) of goldfish IGFI mRNA were designed using the data base from Entrez (NCBI, NIH). PCR amplifications were performed in the linear range with TaKaRa EX Taq HS polymerase. PCR products were electophoresed, and stained with ethidium bromide. Bands for IGF-I mRNA were quantified with the NIH image software (NIH, USA), and quantities were normalized against GAPDH mRNA level.

*2.7. Retinal explant and retinal cell culture*

The retinal explant culture was performed as described previously (Matsukawa et al., 2004a). Briefly, intact (unprimed) or injured (primed) retinas with ON crush 1 or 5 days previously were isolated under sterile condition. Retinal explants were cultured in L-15 medium containing 10% fetal calf serum, on poly-D-lysine-coated 35-mm dishes, at room temperature. An inhibitor of PI3K, wortmannin and IGF-I antibody were added to the culture medium. After 5 days, neurite outlength was observed using phase contrast microscope. The number and length of neurites in all explants were measured. For the retinal cell culture study, explants were treated with 0.25 mg/ml papain and 2.8 mM L-cysteine for 10 min and were dissociated by pipetting. After washing with PBS, they were cultured on poly-D-lysine-coated 12-well plates (Falcon), and were treated with IGF-I (100 nM: Sigma, Germany) and/or wortmannin (100 nM) to study p-Akt activation.

*2.8. Statistics*

Changes in protein levels after ON injury were expressed as percentage increase of control (no treatment). Values were expressed as means ± S.E.M for 3-5 experiments.

Statistical significance was detected by one way ANOVA.

## 3. Results

*3.1. Goldfish RGCs survive after ON crush.* <--- Fig. 1

Since there is no specific marker for goldfish RGCs, we used retrograde labeling with fluorogold to count the number of surviving RGCs before (0 day, Fig. 1A) and after (30 days, Fig. 1B) ON crush. The number of RGCs was constant at 2,000-2,200 RGCs/mm$^2$ during the 30-day treatment (Fig. 1C). No apoptotic cells with DNA fragmentation could be seen during this period (0-30 days) from TUNEL staining (data not shown). Next, we measured caspase-3 activity following ON injury. Caspase-3 activity significantly decreased to 80% of that of the control value 10-20 days after nerve injury, and then gradually returned to control level by 30 days after injury (Fig. 1D). Change of caspase-3 activity in goldfish retinas after nerve injury was only localized in ganglion cells from immunohistochemical studies with anti-caspase-3 antibody (data not shown).

*3.2. Levels of phospho-Akt and Bcl-2 family members in the goldfish retina after ON crush.*

<--- Fig. 2

We investigated phospho-Akt (p-Akt) protein levels in goldfish retinas following ON injury by Western blot analysis. Although total Akt protein level in the retina did not change within 0-30 days (data not shown), p-Akt protein level rapidly increased 3-5 days after nerve crush, peaked by 8 folds 10-20 days after nerve damage, and then gradually decreased 30 days after nerve crush (Fig. 2A). Retinal level of p-Bad protein also rapidly increased 2-6 folds at 3-5 days, peaked at 10-20 days after injury, and then gradually decreased 30 days after ON crush (Fig. 2B). Bcl-2 protein level significantly increased 1.7 folds 10-20 days after injury, and then gradually decreased 30 days later (Fig. 2C), whereas Bax protein level did not change during this period (Fig. 2D).

*3.3. Immunohistochemical study of p-Akt and Bcl-2 in the fish retina after ON crush.*

<--- Fig. 3

For the immunohistochemical study of p-Akt, all nuclear layers were weakly stained in control retinas (Fig. 3A). Optic nerve injury induced a slight increase in p-Akt immunoreactivity in RGCs 3 days (Fig. 3B) and a significant increase 5 days (Fig. 3C) after treatment. For the immunohistochemical study of Bcl-2, all nuclear layers were weakly

stained in control retinas (Fig. 3D). However, immunoreactivity of Bcl-2 drastically increased in the retina, particularly in RGCs 10 days (Fig. 3E), and then gradually decreased by 30 days (Fig. 3F) after ON crush.

*3.4. Temporal changes in IGF-I mRNA and protein levels in the fish retina after ON crush.*

<--- Fig. 4

Total RNA was isolated from retinas at various time points after nerve injury, and changes in amounts of IGF-I mRNA were determined by RT-PCR using goldfish IGF-I primers. An IGF-I band of about 490 bp was obtained. IGF-I mRNA level increased at 1-3 days, peaked by 1.5 folds at 5 days, and then rapidly declined to control level by 10-20 days after injury (Fig. 4A). IGF-I protein level also rapidly increased at 2-3 days, peaked by 2 folds at 5-10 days, and thereafter gradually decreased (Fig. 4B). For the immunohistochemical study of IGF-I, weak immunoreactivity of IGF-I was observed in the inner nuclear layer (INL) and the ganglion cell layer (GCL) of control retina (Fig. 4C). The immunoreactivity of IGF-I slightly increased in the GCL 3 days after nerve crush (Fig. 4D).

*3.5. IGF-I induced activation of Akt and neurite outgrowth in goldfish RGCs.*

<--- Figs 5 & 6

Since IGF-I mRNA and protein levels increased 1-3 days after ON crush, we compared Akt phosphorylation by IGF-I treatment between intact retinas and injured retinas using Western blot analysis(Fig. 5). Akt phosphorylation in the intact (unprimed) retinas was maximally induced by 2.4 folds 2 days after IGF-I treatment, and then returned to control level by 5 days. In contrast, Akt phosphorylation in the injured (primed) retinas with ON crush performed on the previous day peaked by 3.8 folds 1 day after IGF-I treatment, and then returned to control level by 5 days. The activation of Akt was inhibited by the PI3K inhibitor, wortmannin.

To further determine the functional role of IGF-I/Akt system in early stages (1-3 days) of ON regeneration, we investigated effects of IGF-I on neurite outgrowth from retinal explants from adult goldfish. Neurite outgrowth evoked by IGF-I was more significant in primed retinas with ON crush performed on the previous day (Fig. 6D) than that of control (unprimed) retina with (Fig.6C) or without (Fig. 6B) IGF-I (Fig. 6A). Wortmannin significantly attenuated the number of explants with long neurites. Furthermore, addition of

IGF-I antibody (Ab) also significantly inhibited spontaneous neurite outgrowth (Fig. 6A). A large number of explants with long and dense neurites could be spontaneously evoked in primed retinas with ON crush performed 5-7 days ago, but IGF-I did not affect the retina (data not shown).

## 4. Discussion

*4.1. Rapid activation of cell survival signals in the goldfish retina after optic nerve crush.*

RGCs in mammals undergo apoptosis after ON injury, whereas RGCs in lower vertebrates such as goldfish can regenerate their axons (Kato et al., 1999). When considering the regenerative properties of fish RGCs after ON injury, we first paid attention to cell survival/death signals in the goldfish retina. It is most likely that axonal (optic nerve) regeneration allows survival of cell bodies (RGCs). Promotion of cell survival includes 2 concepts: 1) activation of cell survival, and 2) inhibition of cell apoptosis. The PI3K/Akt, Bcl-2 family members, and caspases are well known as survival/death signals. In this study, we chose p-Akt, p-Bad, Bcl-2, Bax, and caspase 3 as representatives of these signals. These molecules are well conserved in almost all phyla from *C.elegans* to human. Protein amounts of these molecules were followed up for 30 days after ON crush. Early changes were upregulation of p-Akt and p-Bad 3-5 days, followed by upregulation of Bcl-2, and downregulation of caspase-3 10-20 days after ON injury (Figs. 1 and 2). Level of pro-apoptotic Bax did not change in any time intervals (Fig. 2). In a previous paper, we

determined 3 stages of goldfish ON regeneration: 1) the early stage (0-6 days) which corresponds to the preparation period for the regeneration process, 2) the middle stage (1-6 weeks) corresponds to an axonal elongation period of the regeneration process, and 3) the late stage (2-6 months) corresponds to the synaptic reinforcement and refinement period of the regeneration process (Matsukawa et al., 2004b; Kato et al., 2006). Therefore, the initial increase in p-Akt (3-5 days) activity might allow survival of RGCs for the preparation of ON regeneration. The sustained increase in p-Akt (5-30 days) activity might allow survival of RGCs for axonal elongation towards the optic tectum. The late increasing activity of Bcl-2 and decreasing activity of caspase-3 might also result in survival of RGCs for axonal elongation (10-20 days). Thus, goldfish RGCs can regrow their axons after ON crush by collaborative works of these molecules. The sequential temporal changes in activities of these cell survival/death signals strongly indicated that the signal cascade started with p-Akt activation, and ended with caspase-3 inactivation during ON regeneration.

*4.2. Promoting role of IGF-I in activation of Akt and axonal regeneration in goldfish RGCs via the PI3K system.*

Optic nerve crush in goldfish induced a rapid 4-5-fold upregulation of p-Akt 3-5 days after injury. This increase continued for 30 days after injury. However, it remains unknown what activates Akt. PI3K plays a central role in cell survival, and phosphorylates Akt (Imai et al., 1999). In the upstream of the PI3K/Akt pathway, some neurotrophic factors activate PI3K. Brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-I (IGF-I) are well known activators of PI3K (Barber et al., 2001; Nakazawa et al., 2002). Both neurotrophic factors and their receptors are localized in cells of the INL and the GCL in goldfish retinas (Boucher and Hitchcock., 1998; Hitchcock et al., 2001). In the present study, we tested the possibility of IGF-I as a p-Akt activator, because Kermer et al. (2000) reported that IGF-I and caspase-3 inhibitor rescued adult rat ganglion cells after ON injury. Furthermore, in a parallel paper (Homma et al., 2007), we clearly showed that IGF-I levels were downregulated in rat RGCs after nerve crush, and neurite outgrowth from adult rat retinas was induced by IGF-I. In the goldfish retina, IGF-I mRNA and protein levels rapidly increased 1 day, peaked 5 days, and then gradually decreased by 20 days after ON crush (Fig. 4). An *in vitro* study further demonstrated that IGF-I maximally activated Akt phosphorylation in primed retinas with ON crush performed on the previous day. Neurite

outgrowth by IGF-I was also maximally activated in the same primed retinas. The one day after ON crush is the same time point for induction of IGF-I mRNA after ON crush. As the activity of these survival factors (IGF-I, p-Akt, p-Bad and Bcl-2) is very weak in the control goldfish retina, the RGCs of goldfish may be resistant to ON crush without elevation of such factors. Although we have no direct evidence of surviving RGCs by IGF-I, the crushing the optic nerve of goldfish certainly induces a rapid upregulation of IGF-I and a subsequent upregulation of p-Akt. These data combined with neurite extension in culture strongly indicate that IGF-I promotes both optic nerve regeneration processes (survival and axonal elongation).

In summary, among of the cell survival signals the earliest molecular event in the regeneration process of the goldfish retina after ON crush involves upregulation of IGF-I (1-3 days), followed by activation of p-Akt (3-5 days), and subsequent upregulation of Bcl-2 and downregulation of caspase-3 (10-20 days) after ON injury (Fig. 7). This sequence of events mirrors the situation in the rat retina, as seen in a parallel paper (Homma et al., 2007). The present data strongly indicate that upregulation of IGF-I is responsible for cell survival of RGCs in goldfish after ON injury through the PI3K/Akt system, and IGF-I directly induces

neurite outgrowth via a wortmannin-dependent mechanism. It is well known that IGF-I also activates the ERK/mitogen activated protein kinase (MAPK) system (Coolican et al., 1997). This should be further evaluated in future studies. Taken together, results from this work and from a parallel paper (Homma and Koriayama et al., 2007) show that IGF-I is one of the turning molecule that primes cells to ON regeneration after injury.

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**Figure Legends**

Fig. 1. Cell survival of goldfish RGCs after optic nerve crush. (A-C). In goldfish retinas, the number of ganglion cells retrogradely labeled with fluorogold was assessed before and after ON crush. Cell number did not change during the 30-day of treatment (B) compared to control retinas (A). Scale bar = 20 μm. A graphical analysis of these results was shown in (C). The experiment was repeated three times (n=3). (D) Caspase-3 activity in the goldfish retina after ON crush. Caspase-3 activity was significantly decreased by 20% of that of the control value 10-20 days after nerve crush. Each value indicates the means ± S.E.M. (n = 3). *P < 0.01 compared to controls (untreated).

Fig. 2. Measurement of retinal levels of cell survival/death proteins in the goldfish retina after ON crush by Western blot analysis. (A) p-Akt. Retinal levels of p-Akt protein increased in the retina 3-20 days after ON injury. (B) p-Bad. Retinal levels of p-Bad protein increased in the retina 3-20 days after ON injury. (C) Bcl-2. Retinal levels of Bcl-2 protein slightly increased from 10-20 days after injury. (D) Bax. Retinal levels of Bax protein did not change during this period (0-30 days). Each value indicates the means± S.E.M. (n = 3) *P < 0.01 compared to controls (untreated).

Fig. 3. Immunohistochemical studies of p-Akt and Bcl-2 in goldfish retinas after ON crush. (A-C). p-Akt. Immunoreactivity of p–Akt weakly increased 3 days (B), and strongly increased 5 days (C) after nerve injury in RGCs compared to control retinas (A). Scale bar = 20 μm. (D-F) Bcl-2. Immunoreactivity of Bcl-2 increased 10 days (E), and gradually decreased by 30 days (F) after nerve injury in RGCs compared to control retinas (D). Scale bar = 20 μm. The experiment was repeated at least three times with the same result.

Fig. 4. Upregulation of IGF-I mRNA and protein in the goldfish retina after optic nerve crush. (A) Early and transient increase of IGF-I mRNA 1-5 days after nerve injury by RT-PCR. Values indicate means ± S.E.M. (n = 3 for each time point). *P < 0.01 compared to control

(untreated) retinas. (B) Transient increase of IGF-I protein 2-10 days after optic nerve injury by Western blot analysis. Values indicate means ± S.E.M. (n = 3 for each time point). *P < 0.01 compared to control (untreated) retinas. (C-D). Immunohistochemical studies of IGF-I in the goldfish retina after optic nerve crush. Note an increase of immunoreactivity in RGCs 3 days (D) after nerve injury compared to control retinas (C). The experiment was repeated at least three times with the same result. Scale bar = 100 μm.

Fig. 5. Induction of Akt phosphorylation in the goldfish retina by IGF-I. In intact, control (intact) retinas (□), Akt phosphorylation was induced by 2.4 folds 2 days after IGF-I treatment. In contrast, in injured retinas with ON crush on the previous day (■), Akt phosphorylation was induced by 3.8 folds 1 day after IGF-I treatment. Wortmannin (■) significantly inhibited Akt phosphorylation of the injured retinas. Values indicate means ± S.E.M. (n = 3). †P < 0.01 compared with 0day intact retinas. *P < 0.01 compared to 0 day injured retinas. #P < 0.01 compared to injured retinas. ‡P < 0.01 compared with 1 day intact retinas.

Fig. 6. Neurite outgrowth induced by IGF-I from goldfish retinal explants through the PI3K/Akt pathway. (A) IGF-I maximally induced neurite outgrowth from primed retinas

with ON crush performed on the previous day compared to unprimed retinas. Wortmannin (WT, 0.1μM) and anti-IGF-I antibody (Ab, 0.1 μg/ml) specifically suppressed neurite outgrowth by IGF-I. *P < 0.01 compared to 1 day control retinas. #P < 0.01 compared to 1 day IGF-I treated retinas. (B-D). IGF-I more effectively induced neurite outgrowth in primed retinas with ON crush performed on the previous day (D) than that in unprimed retinas with (C) or without (B) IGF-I treatment. Scale bar = 200 μm.

Fig. 7. Signal cascade for cell survival in the goldfish RGCs through IGF-I/p-Akt system after ON crush. Initial upregulation of IGF-I and subsequent activations of p-Akt and p-Bad in the retina are followed by the late activation of Bcl-2 protein, and an inactivation of caspase-3 activity. Molecules within solid outlines were examined in this study whereas molecules within dotted outlines were not assayed. The circles show anti-apoptotic (open) or pro-apoptotic (closed) molecules. (+): activate, (↑): increase, (↓): decrease.

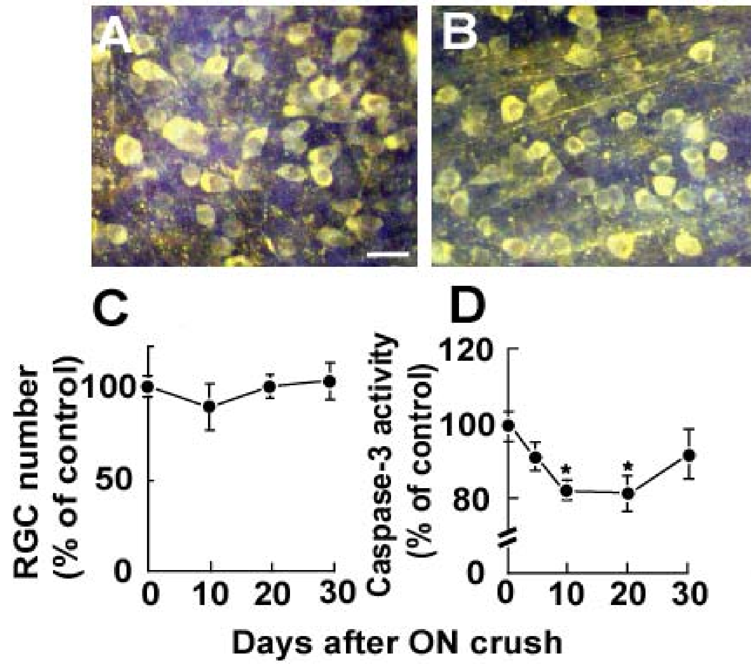


Fig.1 Koriyama et al. 'Neurochemistry International'

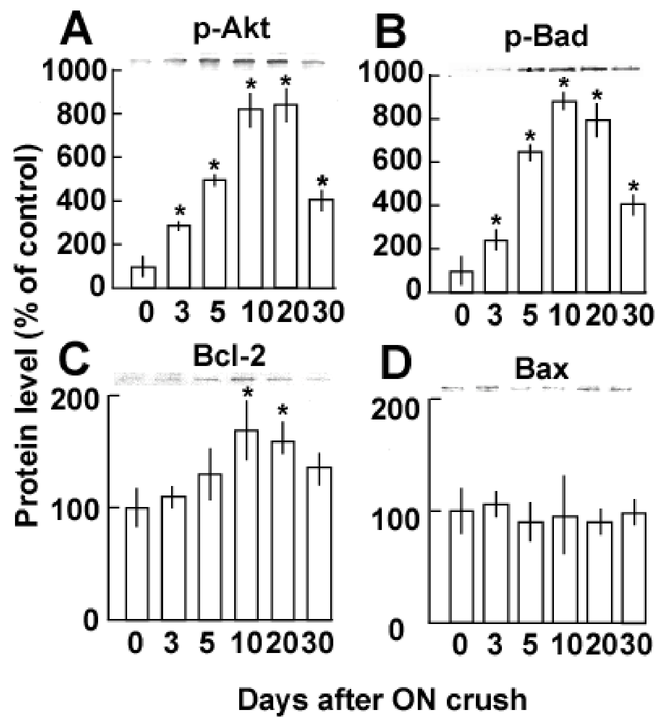


Fig.2 Koriyama et al. 'Neurochemistry International'

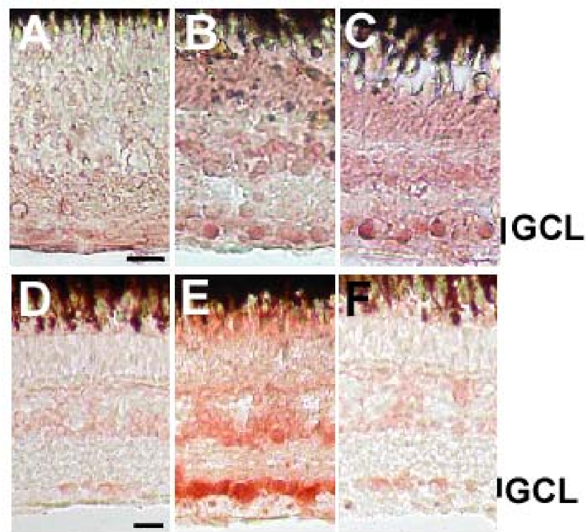


Fig.3 Koriyama et al. 'Neurochemistry International'

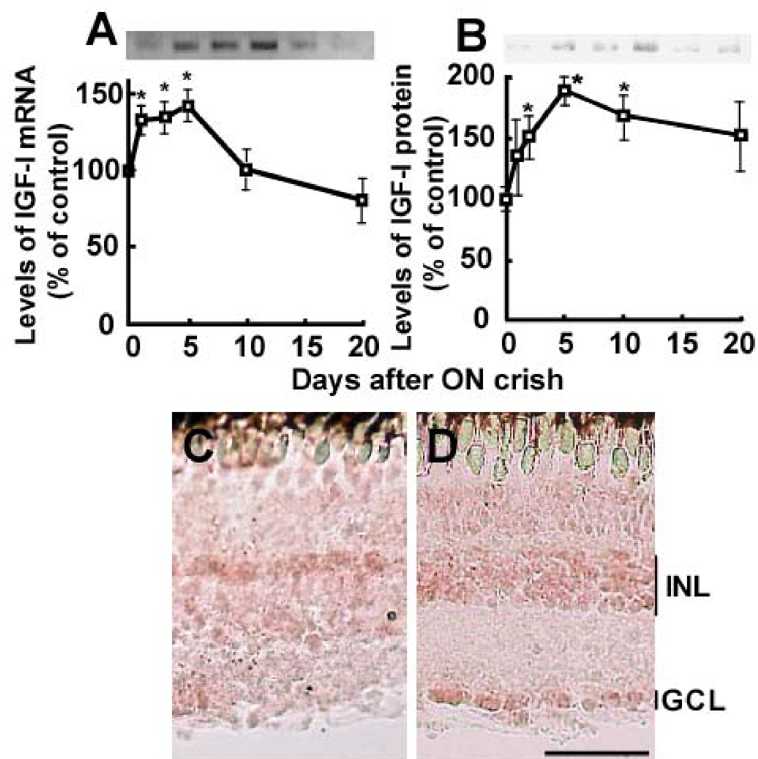


Fig.4 Koriyama et al. 'Neurochemistry International'

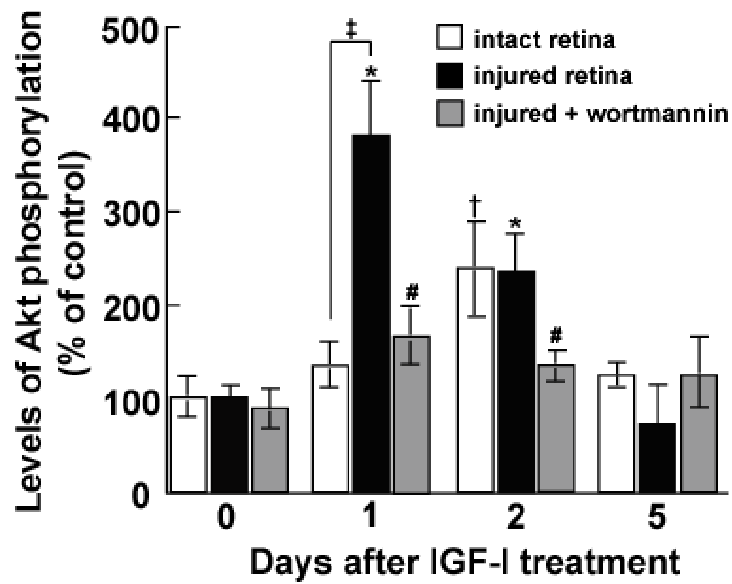


Fig.5 Koriyama et al. 'Neurochemistry International'

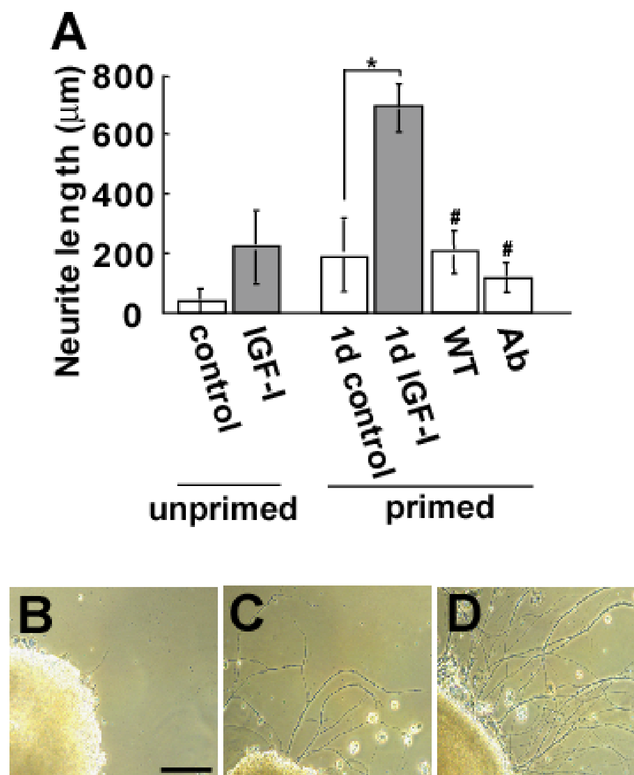


Fig.6 Koriyama et al. 'Neurochemistry International'

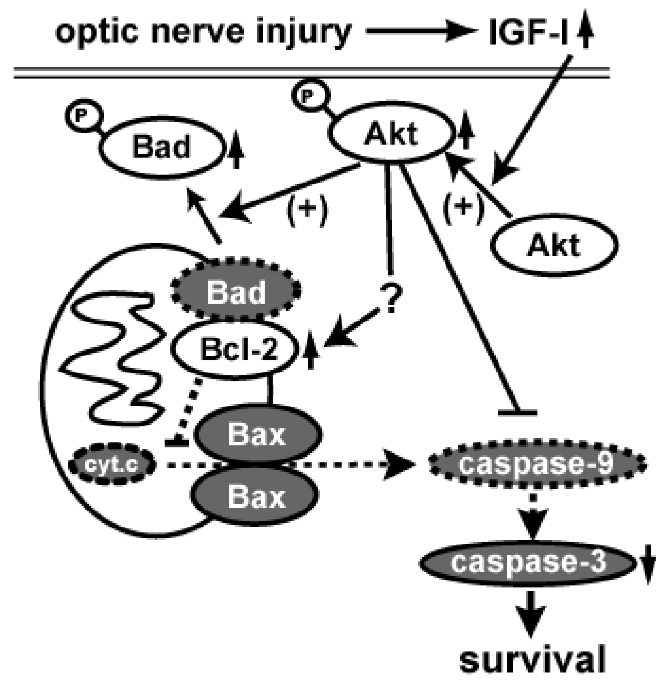


Fig.7 Koriyama et al. 'Neurochemistry International'