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Masahiro, Yamada ; Ryuji, Terayama ; Yoshio, Bando ;
Shinichi, Kasai ; Shigetaka, Yoshida

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Masahiro Yamada [1,2], Ryuji Terayama [1], Yoshio Bando [1], Shinichi Kasai [2], Shigetaka Yoshida [1]

Authors' affiliations

[1] Department of anatomy 1, Asahikawa Medical College, Asahikawa Medical College, Japan

[2] Second Department of Surgery, Asahikawa Medical College, Japan

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Address correspondence to:

Shigetaka Yoshida, MD, PhD

Department of Anatomy 1, Asahikawa Medical College,

Midorigaoka-higashi 2-1-1-1

Asahikawa 078-8510 Japan

Tel: +81-166-68-2300

FAX: +81-166-68-2309

Email: syoshida@asahikawa-med.ac.jp

Abstract

The abdominal sympathetic system is unique in that its postganglionic axons do not directly innervate gastrointestinal smooth muscle layers but exert their effects through the enteric nervous system. The purpose of the present study was to examine the ability of neurons in abdominal sympathetic ganglia to regenerate after axonal injury and to determine whether reinnervation occurs after the removal of ganglia. Axons from the celiac ganglion and superior mesenteric ganglion complex (CG/SMG) of adult female BALB/c mice were crushed or the ganglion complex was removed. Immunohistochemistry, western blotting and in situ hybridization were performed to examine the changes in tyrosine hydroxylase (TH) and growth-associated protein 43 (GAP-43) in the duodenum and the sympathetic ganglia. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling and injection of the tracer dye, fluorogold were also performed. After crushing the nerve, TH in the duodenum disappeared and reappeared within 90 days. In the CG/SMG, TH decreased and increased as in the duodenum, while the expression of GAP-43 changed in the opposite direction. Nerve crushing caused cell death to limited number of neurons in the CG/SMG. The removal of CG/SMG decreased TH in the duodenum and stomach, but 180 days later TH-positive innervation was recovered. Fluorogold injection revealed that the inferior mesenteric ganglion reinnervated the stomach. Therefore, postganglionic sympathetic nerves in the abdomen are able to regenerate and reinnervation occurs even after the removal of sympathetic ganglia.

Keywords:

Celiac ganglion, sympathetic nervous system, ganglionectomy, regeneration, reinnervation

1. Introduction

Postganglionic sympathetic nerves in the abdominal digestive system originate from prevertebral ganglia, namely, the celiac, superior mesenteric ganglion (CG/SMG) and inferior mesenteric ganglion (IMG) complex. The CG/SMG is the main nerve source of the upper digestive tract including the stomach and duodenum. The abdominal sympathetic system is unique in that its postganglionic axons do not directly innervate gastrointestinal smooth muscle layers but exert their effects though the enteric nervous system (Lundgren, 2000). Surgical intervention in the digestive system often accompanies radical dissection of the sympathetic cells and nerves, often causing prolonged diarrhea (Dayton et al., 1984). It is therefore important to recover sympathetic function after neural damage to the digestive system. In other regions, mainly in those innervated by the superior cervical ganglion, adult postganglionic sympathetic neurons exhibit the ability to regenerate (Olson and Malmfors, 1970). Extensive studies have been performed on the regeneration of axons injured by the systemic administration of chemicals such as guanethidine and 6-hydroxydopamine (6-OHDA) (Hokfelt et al., 1972, Evans et al., 1979). High-dose guanethidine disrupts most sympathetic ganglion cells and virtually no regeneration takes place (Evans et al., 1979). The administration of 6-OHDA causes the degeneration of adrenergic terminals only and rapid regeneration occurs (Hokfelt et al., 1972). Nevertheless, little attention has been paid to changes in abdominal sympathetic ganglia in these studies. Regeneration after nerve interruption was observed along the axons of the superior cervical ganglion (SCG) (Purves, 1975, Sato et al., 1980, Handa et al., 1991). However, there have been few studies on the regeneration of postganglionic nerves of the abdominal sympathetic nervous system after mechanical damage (Galligan et al., 1988), which often occurs during clinical interventions (Hill et al., 1985).

Limited literature exists on the neuronal response to nerve injury and subsequent

neuronal changes that occur during sympathetic nerve regeneration. Growth-associated protein 43 (GAP-43) is a key molecule of axonal growth, and its expression reflects the state of regeneration (Benowitz and Routtenberg, 1997). Sympathetic ganglion cells constitutively express GAP-43 (Stewart et al., 1992), but little is known about the expression of GAP-43 in ganglia after injury to sympathetic nerves (Hou et al., 1998, Kato et al., 2003).

Sympathetic ganglia sometimes need to be removed during radical surgery. In such cases, it is more difficult for neurons in alternate ganglia to reinnervate the target tissues because their innervating tissues are often far from the denervated area. Hills et al. interrupted sympathetic nerves along a branch of the mesenteric artery and concluded little or no sprouting from intact adjacent nerves (Hill et al., 1985). Contrary to this, Gloster and Diamond observed the recovery of pilomotor function of the skin after total resection of the distal portion of the innervating sympathetic nerve and concluded that recovery was caused by collateral sprouting (Gloster and Diamond, 1992). It is important to determine whether collateral axons can reinnervate target organs after the removal of abdominal sympathetic ganglia.

To address this, the present study examined the ability of neurons in abdominal sympathetic ganglia to regenerate after axonal injury and to see whether reinnervation occurs after removal of the CG/SMG.

2. Materials and Methods

2. 1. Nerve crush and ganglia removal

Adult female BALB/c mice aged 7-9 weeks were provided by the Animal Institution of Asahikawa Medical College and used in all experiments. All procedures were approved by the Ethical Committee of Asahikawa Medical College.

For CS/SMG nerve crush, the upper abdominal wall of each mouse was cut under anesthesia with pentobarbital (50 mg / kg b.w.), and exposed abdominal viscera were gently pulled out and held in warm saline-soaked gauze. The descending aorta was visualized and sympathetic nerve fibers along the celiac and superior mesenteric arteries were pinched together with arteries using fine forceps. At various times between 1 and 90 days after the operation, mice were killed for histological analysis.

For removal of the CS/SMG, the descending aorta was visualized, the CS/SMG was isolated with fine forceps, severed from the splanchnic nerve trunks with fine scissors, and then surgically excised. The adjacent celiac and superior mesenteric arteries were not damaged during the procedure. At various times between 1 and 180 days after the operation, mice were killed for histological analysis.

2. 2. Histochemistry

The animals were given excess amount of pentobarbital and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The stomach, duodenum and sympathetic ganglia were removed together with the aorta and related arteries. The removed tissues were postfixed in the same fixative, soaked in 30% sucrose in PB and frozen in dry ice powder. Fourteen micrometer sections were cut on a cryostat and put on glass slides coated with 3-aminopropyltriethoxysilane.

For immunohistochemistry, sections were treated with 1% triton X-100 in phosphate buffered saline (PBS, pH 7.4) for 1 hour followed by blocking solution (3%

normal donkey serum and 2% bovine serum albumin (BSA) in PBS) for 1 hour. The primary antibodies used were rabbit anti-GAP-43 (dilution of 1:1000, Santa Cruz, Biotechnology, Santa Cruz, CA) and sheep anti-tyrosine hydroxylase (TH, dilution of 1:1000, Chemicon, Temecula, CA). The sections were incubated with primary antibodies diluted in a solution of 2% bovine serum albumin in PBS. After rinsing with PBS, the sections were incubated with Alexa-488 or Alexa-594 conjugated to a suitable secondary antibody.

In situ hybridization was performed as previously described with GAP-43 cDNA (corresponding to ribonucleotides 143-487 of mouse GAP-43 mRNA, accession no MM008083) as a template for the probe (He et al., 2001). In the double labeling procedure for GAP-43 mRNA and TH protein, immunolabeling to TH was performed prior to the coloring reaction of in situ hybridization. The images of the coloring reaction were digitized and the mean density of randomly selected images was measured using Image J (National Institutes of Health)

Statistical significance was assessed with Student's *t* test.

2. 3. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)

TUNEL was applied to the sections from the CG/SMG at various times after nerve crush. The sections were fixed again with 4% paraformaldehyde in 0.1 M PB, rinsed in 0.1 M PB, treated with 10 μg / ml proteinase K in 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA for 20 min at 37°C, and finally rinsed with PBS. Subsequent TUNEL was performed using a DeadEnd Fluorometric TUNEL System (Promega) according to the manufacture’s instructions. The sections were stained with 1 μl / ml 4',6-Diamidino-2-phenyindole (DAPI, Sigma).

2. 4. Western blot analysis

One to 180 days after removal of the CG/SMG, mice were killed with an excess amount of pentobarbital and their stomachs were removed. They were homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl and 1 mM phenylmethylsulfonyl fluoride, pH 8.0), the homogenate was centrifuged at 19,000 g and the supernatant was used. Thirty micrograms of protein was loaded onto 10% polyacrylamide gel for electrophoresis. After electrophoresis, the separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in 0.1 M Tris buffered saline and 0.05% Tween 20 (TBS-T) for 1 hour at room temperature, and then incubated with 1:500 diluted anti-TH antibody and 1:500 diluted anti-actin antibody (Santa Cruz) in 5% skim milk in TBS-T. After being washed with TBS-T, the blot was incubated with alkaline phosphatase-goat anti-rabbit IgG (BioRad), and then visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

2. 5. Injection of fluorogold

One hundred eighty days after removal of the CG/SMG, 3 mice received fluorogold injected into the anterior wall of the stomach. Five micro litters of fluorogold solution diluted in saline was injected using a Hamilton syringe. During injection, the peritoneal cavity was constantly perfused with saline at 10 ml / min so that fluorogold solution would not be absorbed by tissues outside of the injection site. The abdominal wall was closed 30 min after injection, and two days later the mice were killed for histological analysis.

3. Results

3. 1. Regeneration of CG/SMG axons after nerve crush

To examine the ability of the axons of the CG/SMG to regenerate, the axons along the celiac and superior mesenteric arteries were crushed. TH immunoreactivity was used as a marker of functional postganglionic sympathetic nerves. The duodenum of untreated mice exhibited dense TH-positive networks in the Auerbach and Meissner plexi and perivascular structures (Fig. 1A). TH immunoreactivity decreased 1 day after the nerve crush (Fig. 1B) and reached a peak decrease after 3 days. Even 30 days postoperative, virtually no immunoreactivity was observed (Fig. 1C). However, 90 days after the operation, TH immunoreactivity reappeared (Fig.. 1D).

3. 2. Neuronal responses in the CG/SMG after nerve crush

Neuronal cell bodies in the CG/SMG also exhibited degenerative and regenerative responses to nerve crush. Abundant expression of TH in the control CG/SMG (Fig. 2A) was significantly decreased at 14 days (Fig. 2E). Although the expression of TH 30 days after the operation remained significantly lower than the control, the expression recovered after 90 days (Fig. 2C,D and E). This time course was comparable to the change in TH expression in the duodenum.

GAP-43 and its mRNA exhibited signs of regeneration. The control CG/SMG expressed an intermediate amount of GAP-43 (Fig. 3B) and its mRNA (Fig. 3A), and 1 day postoperative, GAP-43 expression was decreased while its mRNA expression was increased. GAP-43 and its mRNA were increased and peaked 7 – 14 days postoperative (Fig. 3C and D), then gradually decreased, and 90 days later the expression returned almost to the control level (Fig. 3E and F). The overall density of GAP-43 mRNA reaction was measured and shown in Fig. 3G. Double labeling of TH and GAP-43 mRNA revealed that GAP-43 mRNA-positive cells were largely devoid of TH

immunoreactivity after nerve crush (Fig. 3C), while TH and GAP-43 mRNA were colocalized in the control and regenerated CG/SMG (Fig. 3A and E). TUNEL revealed neuronal death after CG/SMG nerve crush. Twelve hours after the operation, no TUNEL-positive cells were observed. A maximum of 7 TUNEL-positive cells per section appeared 24 and 48 hours after the operation (Fig. 4B), but 4 days onward no TUNEL-positive cells were observed. This result indicates that cell death occurs transiently and that the number of death is rather small.

3. 3. Reinnervation occurs 180 days after the removal of the CG/SMG

One day after CG/SMG removal, TH immunoreactivity in the duodenum decreased significantly (Fig. 5B), as was the case for nerve crush, and did not recover until 90 days postoperative (Fig. 5C). However, 180 days after the operation, TH immunoreactivity recovered nearly to the control level (Fig. 5D). Similar changes were observed in the stomach, jejunum and ileum (data not shown). We analyzed the time course of the quantity of TH present in the stomach by western blotting (Fig. 5E). Consistent with immunohistochemistry, the quantity of TH significantly decreased one day after surgery and recovered 180 days later (Fig. 5E).

3. 4. IMG neurons may reinnervate the stomach after removal of the CG/SMG

We next attempted to determine which ganglion cells reinnervate the stomach after denervation by removal of the CG/SMG. Fluorogold, a retrograde tracer, was injected into the anterior wall of the stomach 180 days after CG/SMG removal. In unoperated control mice, labeled neurons were observed in the CG/SMG (Fig. 6A), and in the IMG we could observe no labeling but only intrinsic fluorescence (Fig. 6B) as was also observed in the IMG without fluorogold injection (Fig. 6D). In contrast, labeled neurons were observed in the IMG of mice that had their CG/SMG removed 180 days before

fluorogold injection (Fig. 6C). Special care was taken to observe remnant CG/SMG in these mice, but no fluorogold-labelled neurons were observed around the celiac or superior mesenteric arteries. FG immunoreactivity was further confirmed by immunolabeling with anti-FG antibody. The IMG after removal of the CG/SMG with FG injection was labeled with anti-FG antibody, whereas the IMG without FG injection (Fig. 6F) or the IMG with injection of FG but without removal of CG/SMG (Fig. 6G) was not stained.

4. Discussion

The enteric sympathetic system is unique in that its ganglia are prevertebral and its postganglionic axons indirectly exert their effects through the enteric nervous system. However, less attention has been paid to their regeneration capacity, partly because the CG/SMG is relatively difficult to access in animal experiments. Long-term observations in the present study enabled us to determine that the enteric sympathetic nervous system regenerates after axonal damage and reinnervates target organs even after ganglionectomy in mice.

After nerve crush, the expression of TH and GAP-43 in sympathetic ganglia changed in the opposite directions, namely increased GAP-43 and decreased TH in the early on, while GAP-43 decreased and TH expression was recovered during regeneration. Only a few studies exist on change in GAP-43 expression in sympathetic ganglia after postganglionic axonal injury (Hou et al., 1998, Kato et al., 2003). The present study combining immunohistochemistry and in situ hybridization showed that ganglionic cells increase their production of GAP-43 during regeneration and that when regeneration is complete this expression returns to constitutive levels. Contrary to GAP-43, decreases in the expression of molecules required for synaptic transmission occur after injury (Kessler and Black, 1979, Koo et al., 1988). TH decreases 2 to 10 days after the transection or crush of postganglionic nerves in the rat SCG (Koo et al., 1988). The present results show that the TH expression in the cell body is recovered a relatively long time after injury. Thus, in the early phase, surviving neurons regenerate with high GAP-43 and low TH, and in the late phase TH expression is recovered with low GAP-43 after a connection has been reestablished.

The present study is the first report to show the ability of the sympathetic ganglion in adults to reinnervate organs that were not its target before injury. The IMG usually innervates the lower digestive systems including the transverse and descending colons

but not the stomach or duodenum (Fig. 6B) (Furness and Costa, 1987). However, injection of fluorogold into the stomach labeled neurons in the IMG a relatively long time after removal of the CG/SMG, which normally innervates the stomach and duodenum. This indicates that neurons in the IMG might send collaterals that sprout from axons nearby cell bodies rather than those that sprout from the nearby distal end of axons as a possible mechanism for organ reinnervation. Contradictory observations have been made regarding the collateral sprouting of postganglionic sympathetic nerves. Gloster and Diamond observed collateral sprouting from nerves that innervate pilomotor smooth muscles in the skin (Gloster and Diamond, 1992), while Hill et al. concluded that there is only limited sprouting from existing noradrenergic nerves as seen by fluorescent histochemistry (Hill et al., 1985). Kato et al. observed increased GAP-43 mRNA in the ganglion adjacent to the injury, namely, the major pelvic ganglion contralateral to the injured pelvic nerve (Kato et al., 2003). The present study indicates that noradrenergic neurons are able to sprout axonal collaterals. One possible interpretation of the observation made by Hill et al. is that catecholaminergic vesicles may be temporarily absent in the terminals during collateral sprouting, since we observed reduced TH immunoreactivity during regeneration. It is a well-known fact that regenerating neurons temporarily reduce the expression of transmitters during regeneration. Kato et al. also observed the reduced number of TH-immunoreactive cells among GAP-43 mRNA-expressing neurons in the major pelvic ganglion contralateral to the injured pelvic nerve (Kato et al., 2003).

Technical comments

We crushed postganglionic sympathetic nerves by pinching arterial walls. This method may only damage axons, sparing neural cell bodies in the ganglia in comparison with chemical injury (Liu et al., 1996). However, pinching might only damage a part of

the nerve. Nevertheless, a large part of the target organ and ganglia were affected. Immunoreactivity for TH disappeared in all sections of the duodenum (Fig. 1) and stomach (data not shown) to the same extent as for removal of the CG/SMG. The CG/SMG ganglia also reduced their expression of TH and increased the expression of GAP-43 and its mRNA (Fig. 2, 3), indicating that most cells and axons were affected.

The present method using fluorogold injection was carefully designed. In preliminary experiments without continuous washing of the peritoneal cavity with saline, some leakage and absorption of fluorogold could not be prevented, resulting in the labeling of a large number of cells of unrelated ganglia in the normal mouse (unpublished observation) (Skirboll et al., 1989). Careful and thorough washing allowed us to examine neurons that innervated the target organs without labeling unrelated cells (Fig. 6). There remains small possibility that the peritoneum somehow changed its characters by the operation and became hyper-permissive and leaked dye reached the IMG in spite of our careful method. Although further studies will be necessary to address this point, we assume it is unlikely 6 months after the removal.

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Figure legends

Figure 1 Changes in TH immunoreactivity in the duodenum after nerve crush. The duodenum of untreated mice exhibited dense TH-positive networks in the Auerbach and Meissner plexi and perivascular structures (arrows in A). TH immunoreactivity decreased 1 day after the nerve crush (B) even 30 days postoperative virtually no immunoreactivity was observed (C). At 90 days, TH immunoreactivity reappeared (D). Scale bar = 200 μm.

Figure 2 Changes in TH immunoreactivity in the CG/SMG after nerve crush. The expression of TH in the CG/SMG of a control mouse (A) and that in the CG/SMG 14 days (B), 30 days (C) and 90 days (D) after the nerve crush. E, quantitative analysis of TH-immunoreactive cells in the CG/SMG before and after the nerve crush. Asterisks indicate significant difference from the control ($p < 0.01$). Scale bar = 100 μm.

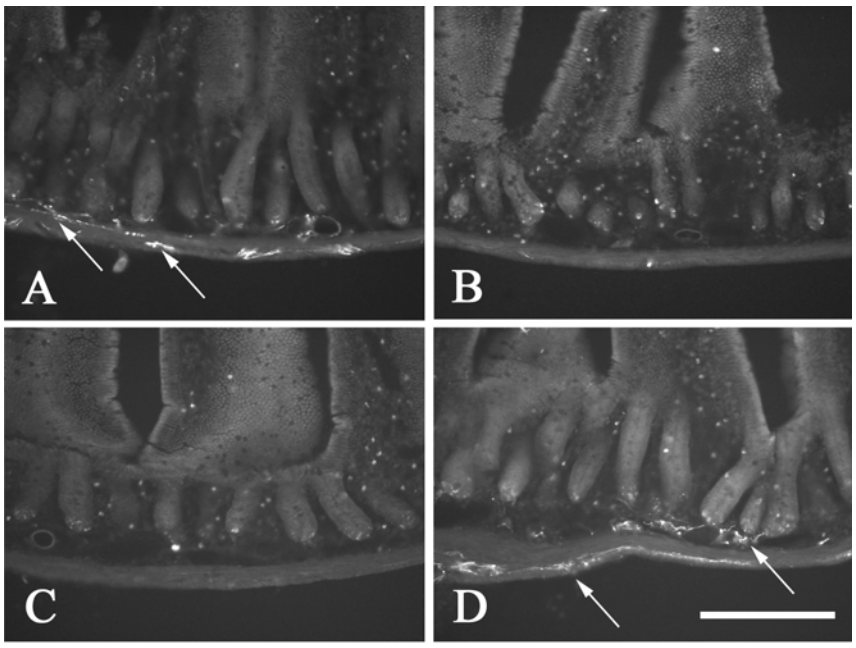
Figure 3 GAP-43 and its mRNA in the CG/SMG after nerve crush. Double labeling with GAP-43 mRNA (brown) and TH (orange) of CG/SMG of control mice (A) and mice 7 days (C) and 90 days (E) after the nerve crush. Control CG/SMG expressed an intermediate amount of GAP-43 protein (B) and abundant GAP-43-positive structures were observed 7 days after the nerve crush (D). GAP-43 expression returned to control levels after 90 days (E). G, quantitative analysis of GAP-43 mRNA-positive cells in the CG/SMG before and after nerve crush. Asterisks indicate significant difference from the control ($p < 0.05$). Scale bar in E =100 μm (also applied to A and C); scale bar in F = 50 μm (also applied to B and D).

Figure 4 TUNEL staining after CG/SMG nerve crush. In the control CG/SMG, no TUNEL positive cells were observed (A). Twenty-four hours after nerve crush,

TUNEL-positive cells were observed in CG/SMG (arrows in B). Scale bar = 50 μm.

Figure 5 Changes in TH immunoreactivity in the duodenum after removal of the CG/SMG. The duodenum of untreated mice exhibited dense TH-positive networks in the Auerbach and Meissner plexi and perivascular structures (arrows in A). TH immunoreactivity decreased 1 day after the removal (B), and even 90 days, postoperative virtually no immunoreactivity was observed (C). One hundred eighty days postoperative, TH immunoreactivity reappeared (D). Western blot analysis shows the change in TH in the stomach after removal of the CG/SMG (E). The stomach was removed on the days indicated after removal of the CG/SMG and 30 μg of protein was put onto each lane. Beta-actin was probed as an internal control. Scale bar = 200 μm

Figure 6 Reinnervation of the stomach was established by IMG. Fluorogold was injected into the anterior region of the stomach of control mice (A, B and D) and mice whose CG/SMG was removed 180 days beforehand (C). In control animals, a population of CG/SMG neurons exhibited distinct fluorogold labeling (arrows in A), while some cells exhibited weak fluorescence (arrowheads) as in the IMG (arrowheads in B), which was comparable to cells in the negative control (D). Many fluorogold-labeled cells were observed in the IMG 180 days after removal of the CG/SMG (arrows in C). The boxed area in C is enlarged in the inset. The accumulation of FG was further confirmed by FG-immunoreactivity (E-G). FG-immunopositive cells were observed in the IMG after removal of the CG/SMG. In the control IMG with injection of FG in the stomach (F) or without injection of FG (G) did not show positive cells. Scale bars = 100 μm.



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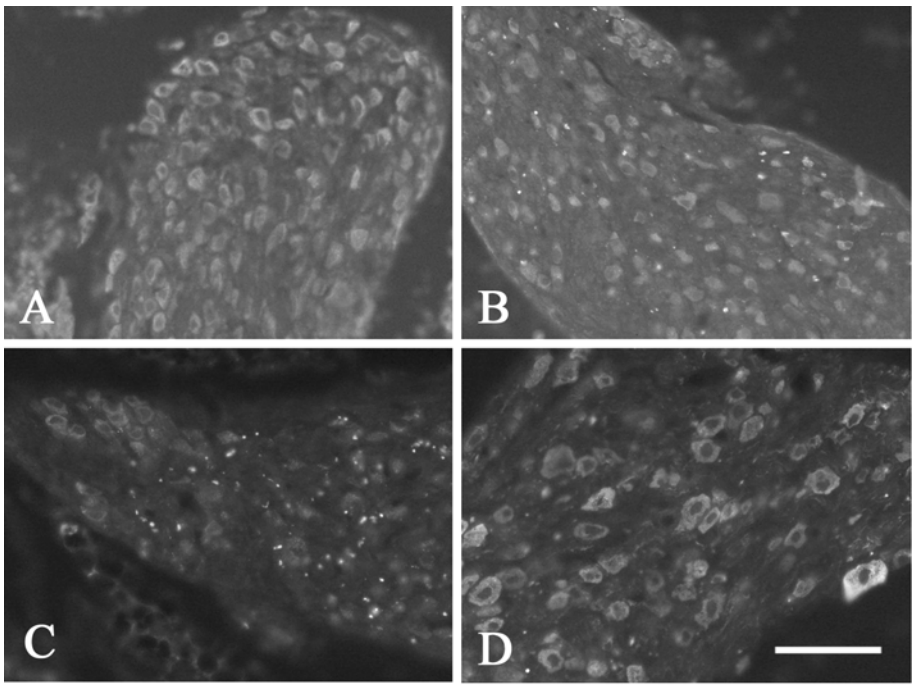

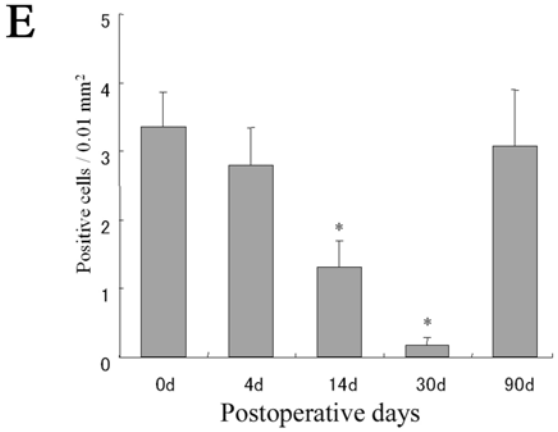

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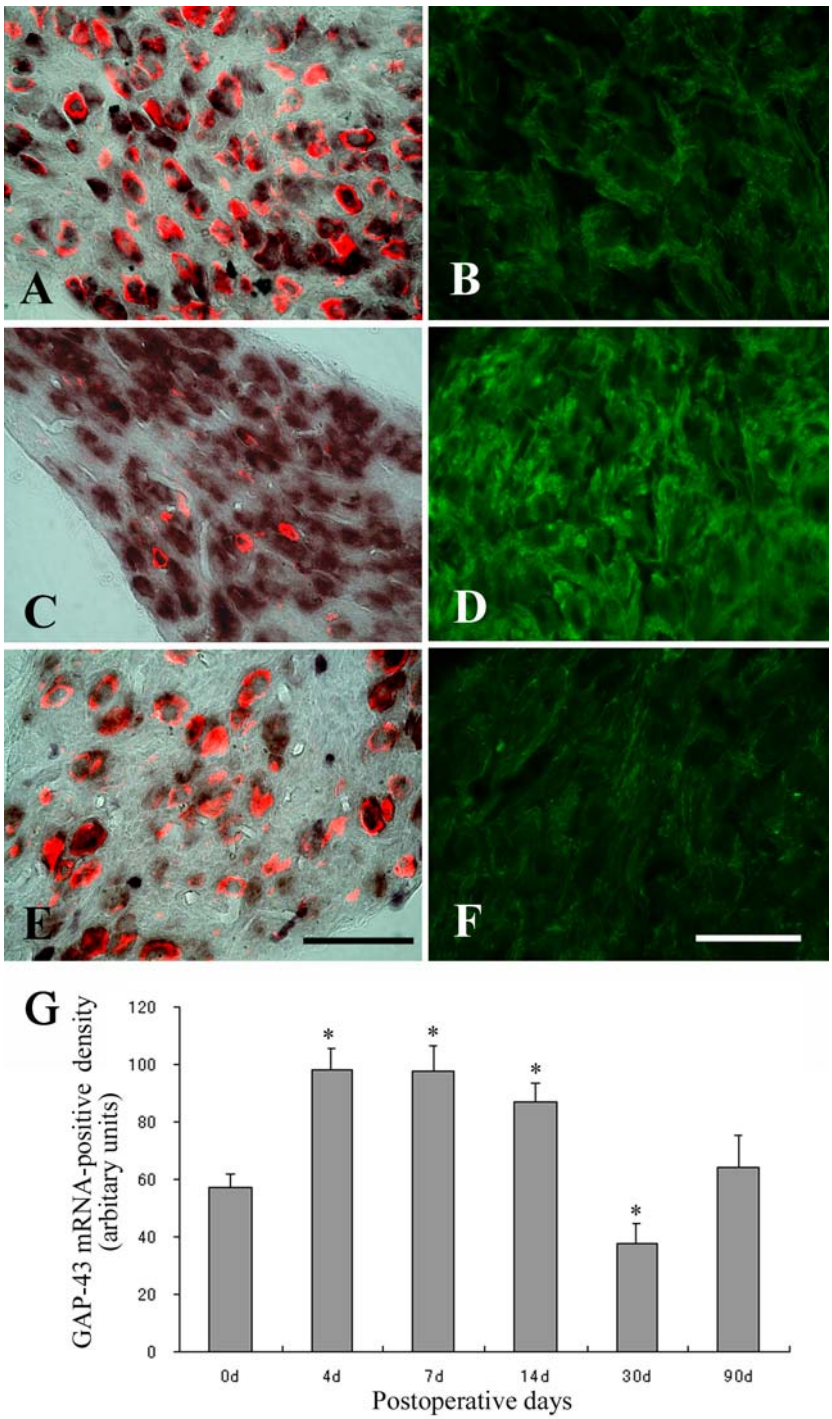

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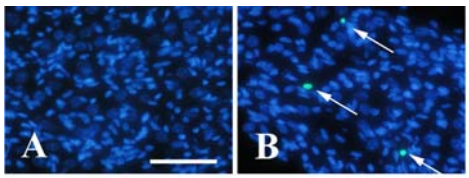

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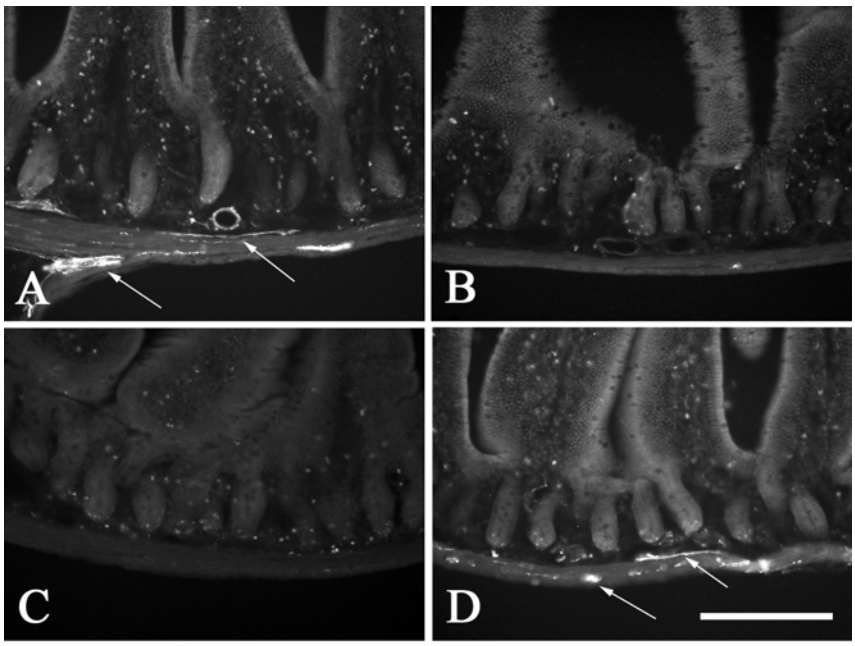

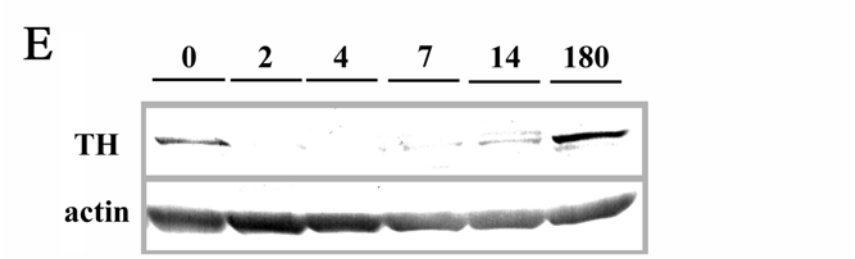

Fig. 5 Yamada et al.

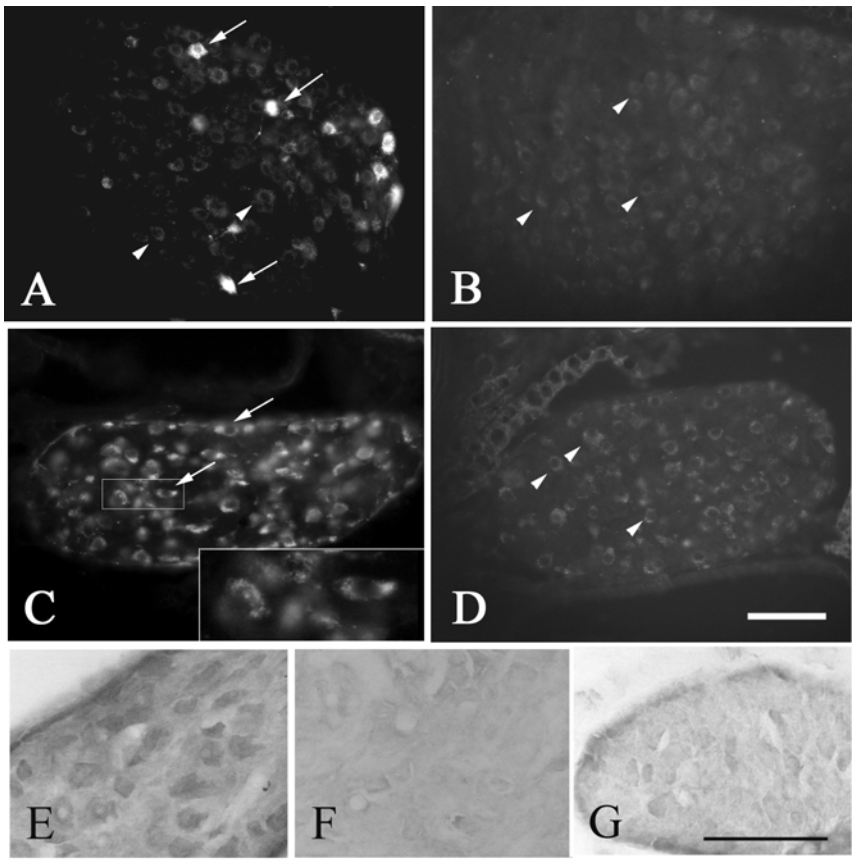

Fig. 6 Yamada et al.