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Immunohistochemical study on GABAergic system in salivary glands

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Abstract

Gamma-aminobutyric acid (GABA) and its receptors are found in the central nervous system and several peripheral tissues. The purpose of this study was to determine the expression and distribution of GABA and glutamate decarboxylase (GAD), a GABA biosynthetic enzyme, in rat salivary gland. Western blot and real time quantitative RT-PCR revealed that GAD67 was the major isoform of GAD in the salivary glands. Furthermore, both GABA and GAD were detected around the acinar cells in the submandibular glands by immunohistochemical analysis. When both sympathetic and parasympathetic nerves related to the submandibular glands were denervated, the immunoreactivities of GABA and GAD were dramatically depressed, and levels of GAD67 and GABA significantly decreased. However, no morphological changes in the glands were observed after denervation. These results indicate that GAD67 is present around acinar cells in the salivary glands, and suggest that the GABAergic system in the glands is closely related to the autonomic nervous system.

Keywords: gamma-aminobutyric acid; glutamate decarboxylase; salivary gland; peripheral nervous system; innervation

1. Introduction

Gamma-aminobutyric acid (GABA) is a major neurotransmitter in the central nervous system. It is synthesized from glutamate by a glutamate decarboxylase (GAD) and metabolized to succinic semialdehyde by a GABA transaminase. GABA exerts its actions via ionotropic receptors GABA_A and GABA_C and metabotropic receptor GABA_B (Chebib and Johnston, 1999; Bormann, 2000). GABA and its receptors are not limited to the central nervous system, but are also found in several non-neural tissues such as the kidney, pancreas, and testis (Ritta and Calandra, 1986; Sorenson et al., 1991; Fujimura et al., 1999; Watanabe et al., 2002).

In an earlier study, GABA and its biosynthetic enzyme, GAD, were detected in rat salivary gland and brain (Sawaki et al., 1995). Moreover, the GABA_A receptor has been reported to be localized in rat salivary gland, and GABA has been shown to inhibit salivary secretion via GABA_A receptors (Shida et al., 1995; Kawaguchi and Yamagishi, 1996). These findings suggest that the GABAergic projection system is present in the salivary glands, and that it is involved in salivary secretion. However, the histochemical evidence for this remains to be studied in detail.

Mammalian species express two isoforms of GAD, GAD67 and GAD65, which are products of distinct genes. The GAD65 gene is located on chromosome 10, and the GAD67 gene on chromosome 2 in human (Bu et al., 1992). These isoforms differ markedly in terms of expression level and physiological role. For example, GAD65 is preferentially localized near the neuronal synaptic vesicles, whereas GAD67 is more widely distributed within the cells (Soghomonian and Martin, 1998). In this study, we performed a detailed immunohistological study and Western blot to determine the distribution of GABA and GAD in rat salivary gland. Moreover, we investigated the relationship between the GABAergic system and the

autonomic nervous system in these glands.

2. Materials and methods

2.1. Materials

The chemicals used in this study were purchased as follows: Gabaculine from Sigma (St. Louis, MO, USA); Hoechst 33342 from Wako Pure Chemical Industries (Osaka, Japan); anti-GABA polyclonal antibody and anti-GAD polyclonal antibody from Chemicon (Temecula, CA, USA); anti-rabbit-IgG-horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Enhanced Chemiluminescence (ECL) Plus system from GE Healthcare Biosciences (Little Chalfont, Bucks, UK); and Alexa Fluor 488 goat anti-rabbit IgG from Invitrogen Corp. (Carlsbad, CA, USA). All other reagents used were of analytical grade.

2.2. Animals

Male Wistar rats weighing 230-250 g were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The animals were kept at a constant room temperature ($21^{\circ} \pm 2^{\circ}\text{C}$) under a 12-h light-dark cycle (lights on between 6:00 am and 6:00 pm) and maintained on commercial laboratory chow and tap water for at least 5 days before being used. All efforts were made to minimize the number of animals used and suffering. All experiments complied with the Guidelines for the Treatment of Experimental Animals approved by The Japanese Pharmacological Society and Tokyo Dental College.

2.3. Administration of gabaculine

Gabaculine at 50 mg/kg in a volume of 0.1 ml per 100 g body weight was intraperitoneally administered in each rat. Gabaculine was dissolved in physiological saline and prepared immediately before use. Control rats were given an equal volume of physiological saline.

Histochemical examination was performed 6 h after administration of gabaculine.

2.4. Denervation

Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and denervation was carried out as described previously (Yao et al., 2005), with some modifications. Briefly, sympathetic and parasympathetic denervations were performed by removing the superior cervical ganglion and cutting the chorda tympani nerve, respectively. All surgeries were performed on the left side. The experiment was performed 14 days following this procedure. To confirm that denervation was successful, we measured the weight of the submandibular gland on the experimental and control sides after the animal had been killed for experiments as reported previously (Yao et al., 2005). In this earlier study, gland weight was reduced by 10-20% at 7 days after the operation. In this study, none of the rats died during the 14-day period between surgery and the commencement of experiments.

2.5. Immunohistochemistry

Rats were transcardially perfused with ice-cold 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline (pH 7.4) under deep pentobarbital anesthesia. The salivary glands were fixed in the same fixative at 4°C overnight. For histological assessment of the submandibular glands, paraffin-embedded sections (10 µm) were stained with hematoxylin/eosin and Oil red-O. To determine GABA, the glands were embedded in paraffin and sectioned to a thickness of 10 µm. After deparaffinization, the sections were blocked with 2% normal goat serum for 2 h. To determine GAD, the glands were successively soaked in ice-cold 10, 20 and 30% sucrose in phosphate-buffered saline for cryoprotection, and cut into 10-µm sections using a cryostat-microtome (Leica, Deerfield, IL, USA). The cryostat sections were dried and fixed with 0.3% H₂O₂ and 40% methanol in phosphate-buffered saline for 30

min. For permeabilization and blocking, the sections were pretreated with 0.1% Triton X-100 and 2% normal goat serum in phosphate-buffered saline. These blocked sections were incubated with anti-GABA antibody (1:400) or anti-GAD antibody (1:500) overnight at 4°C. After washing, these sections were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG antibody (1:500) for 2 h at room temperature. Cell nuclei were stained with Hoechst 33342 (50 µg/ml). Fluorescence imaging was carried out with the Axiophot 2 (Carl Zeiss, Oberkochen, Germany).

2.6. Western Blot

Rat salivary glands and other organs were quickly excised and homogenized in ice-cold lysis buffer containing 25 mM Hepes (pH 7.4), 10 mM EGTA, 10 mM EDTA, 50 mM NaCl, 1% TritonX-100, 0.1% SDS, 1% sodium deoxycholate, 20 mM 2-mercaptoethanol, and protease inhibitors. The lysates were separated by 7.5% SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes as described previously (Kosuge et al., 2003). The membranes were treated with primary antibody (1:1,000) at 4°C overnight and then incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1 h. Immunoreactivity was detected with the Enhanced Chemiluminescence (ECL) Plus system (GE Healthcare Biosciences, UK). Optical density of the blots was measured with the Scion imaging software (www.scioncorp.com).

2.7. Measurement of GABA

Measurement of GABA was performed by high-pressure liquid chromatography as reported previously (Sawaki et al., 1995). After denervation, the submandibular glands were excised and homogenized in ice-cold methanol. The homogenate was centrifuged at 15,000 g for 10 min at 4°C, and the supernatant obtained was mixed with *o*-phthalaldehyde and injected into a

high-pressure liquid chromatography system. Next, 0.05 M sodium phosphate (pH 3.5) containing 50% methanol was delivered at a flow-rate of 0.8 ml/min. The fluorescence of the GABA eluate was measured spectrofluorometrically at an emission wavelength of 445 nm, and at an excitation wavelength of 340 nm. 2-Aminopentanoic acid was used as an internal standard.

2.8. Extraction of total RNA and real time quantitative RT-PCR

Total RNA was extracted using the NucleoSpin[®] RNA II Purification Kit (Macherey Nagel, Düren, Germany). RT-PCR was carried out according to the manufacturer's protocol, using 5.0 µg total RNA and the DNA Engine Opticon 2 (MJ Research, MA, USA) (Yoshikawa et al., 2004). The following primers were used for PCR amplification: [5'-ACCGGATTGGATATGGTTGGATTAG-3' and 5'-CCTGGCCAGCCAATGATTTTC-3' for GAD65 (146 bp); 5'-ATGGGTGTGCTGCTCCAGTG-3' and 5'-GTCATACTGCTTGTCTGGCTGGAA-3' for GAD 67 (111 bp); 5'-TGTCACCAACTGGGACGATA-3' and 5'-ACCCTCATAGATGGGCACAG-3' for β-actin (279 bp)]. PCR amplification conditions consisted of 35 cycles (94°C for 15 s, 60°C for 30 s, 72°C for 15 s, followed by final extension for 10 min at 72°C).

2.9. Measurement of Protein Concentration

Protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

2.10. Statistical Analysis

Data were analyzed using the Student t-test when only two groups were involved. In the remaining cases, the results were statistically analyzed using the Tukey test after performing an ANOVA. All data are expressed as mean ± S.E.M. A *P* value of less than 0.05 was

considered to be statistically significant.

3. Results

3.1. Expression of GAD67/65 in salivary gland

To detect GAD isoforms, we designed specific primers according to their sequences (Geigerseder et al., 2003). The mRNAs of GAD67 and 65 were strongly detected in cerebral cortex and cerebellum, with the expression level of GAD67 mRNA being significantly higher than that of GAD65 (Figs. 1A and B). Although the levels were much lower than those in the brain, the PCR product from GAD67 mRNA was also detected in all salivary glands examined, whereas GAD65 mRNA was not expressed (Figs. 1A and B). Their levels were approximately $1.9\text{-}2.1 \times 10^{-4}\%$ of those in the cerebral cortex, but there was no significant difference between the three salivary glands. Correspondingly, Western blot also revealed endogenous GAD67 protein in all salivary glands, although the levels were 1.8-1.9% of those in the brain (Figs. 1C and D). No expression of GAD65 protein was detected in any of the salivary glands (Fig. 1C).

3.2. Distribution of GAD67 in salivary gland

The submandibular gland mainly consisted of serous acini and all types of duct. We examined the distribution of endogenous GAD67 protein in rat submandibular gland using immunohistochemical analysis. Immunoreactivity of GAD67 protein was strongly detected around acinar cells (Fig. 3A). In order to investigate the correlation between the GABAergic system and the autonomic nervous system, the sympathetic and parasympathetic nerves related to the submandibular gland were denervated. The operation resulted in a reduction of the weight of submandibular gland similar to that in a previous report (Yao et al., 2005). Weight loss was approximately 10% at 14 days after denervation, which was not significant

(Fig. 2A). However, no morphological change in the glands was observed by staining with hematoxylin/eosin and Oil red-O (Fig. 2B). On the other hand, immunoreactivity of GAD67 around the acinar cells of the submandibular glands was dramatically depressed by denervation, with intensity decreasing to 12.8% from 111.7% of the control value (Figs. 3A and 3B). Moreover, levels of endogenous GAD67 protein were significantly decreased to approximately 56% of the control values, but not levels of β -actin protein (Fig. 3C).

3.3. Distribution and content of GABA in salivary gland

We further measured distribution and content of GABA in submandibular gland after denervation. Immunoreactivity for GABA was detected around the acinar cells in the control glands, with this activity showing a significant increase with administration of gabaculine (50 mg/kg, i.p.), which is a GABA transaminase inhibitor that increases GABA concentration (Fig. 4A). As expected, the increase in GABA immunoreactivity induced by gabaculine showed a significant decrease with denervation, with intensity decreasing to 3.6% from 129.4% of the control value (Figs. 4A and 4B). Correspondingly, the concentration of GABA in the submandibular gland was found to have significantly decreased to approximately 57% of the control values by denervation (Fig. 4C).

4. Discussion

In this study, we investigated the expression and distribution of GAD67 and 65 in rat salivary gland. Real time quantitative RT-PCR revealed that the isoform of GAD in the salivary gland was GAD 67, not GAD 65 (Fig. 1A). Correspondingly, the GAD67 protein was detected in the salivary gland using specific GAD67/65 antibody, whereas the GAD65 protein was not (Fig. 1C). GAD67 in brain is responsible for synthesis of >90% of GABA, whereas the major GAD in pancreatic B cells is the GAD65 isoform (Christgau et al., 1992; Watanabe et al.,

2002). These are just two examples of tissue heterogeneity in terms of GAD isoform distribution. The kinetic properties of GAD in rat salivary gland have previously been reported to be similar to those of brain GAD (Sawaki et al., 1995). These results suggest that the major GAD in rat salivary gland is the GAD67 isoform.

In terms of distribution within the submandibular gland, GAD67 and GABA were localized around the acinar cells (Figs. 3A and 4A). This result is consistent with earlier results on the distribution of the GABA_A receptor, which was found in acinar and duct cells in rat submandibular gland (Shida et al., 1995). These results also suggest that GABA is synthesized by GAD67 around the acinar cells in salivary gland, and that this synthesized GABA acts on GABA_A receptors in acinar cells.

Benzodiazepines such as diazepam cause a decrease in salivary secretion from the salivary glands in humans and animals (Sreebny and Schwartz, 1986). Benzodiazepine receptors are classified into two types: a central-type, coupled to the GABA_A receptor, and a peripheral-type, not linked to the GABA_A receptor (Tallman et al., 1978; Verma and Snyder, 1989). Previous reports showed two types of benzodiazepine receptor in salivary gland (Yamagishi and Kawaguchi, 1998; Yamagishi et al., 2000). Benzodiazepines also suppressed the release of amylase and phosphatidylinositol turnover in rat parotid cells, and their inhibition was blocked by benzodiazepine receptor antagonists (Okubo and Kawaguchi, 1998; Kujirai et al., 2002). These findings indicate that benzodiazepines and/or GABA not only suppress the central nervous system, but also act directly on the salivary glands, and that the GABAergic system in the salivary glands is linked to inhibitory responses of salivary secretion. Our findings strongly suggest that GAD67 and GABA around acinar cells are involved in the suppression of salivary secretion.

We found that protein and mRNA expression of GAD67 in salivary gland was much lower than that in brain (Fig. 1). These results suggest that GAD67 in salivary gland is distributed in the neural terminals. The GAD67 protein has been utilized as a marker of GABAergic neurons (Esclapez et al., 1994). Therefore, we investigated the correlation between the GABAergic system and the autonomic nervous system in the salivary gland by denervation, and measured the weight of the gland to confirm whether the operation was successful. When both sympathetic and parasympathetic nerves related to the submandibular gland were denervated, gland weight decreased by approximately 10% at 14 days after surgery (Fig. 2A). This decrease was consistent with the result reported previously (Yao, et al., 2005), and was reflected in the successful denervation. It seems that the weight loss observed was only a partial effect, as no morphological changes in the glands were observed after denervation (Fig. 2B). However, the immunoreactivities of GAD67 and GABA in the acinar cells of the submandibular gland were dramatically depressed after denervation (Figs. 3A and 4A). Moreover, the levels of endogenous GAD67 and GABA in the submandibular gland were also significantly decreased by denervation (Figs. 3C and 4C). These results suggest that the GABAergic system in the submandibular gland is closely related to the autonomic nervous system. In immunohistochemical studies, it has been demonstrated that GABA- or GAD-immunoreactivity and acetylcholinesterase activity were colocalized in numerous nerve fibers and some chromaffin cells (Hallanger et al., 1986; Fisher et al., 1988; Gritti et al., 1993; Iwasa et al., 1999). Furthermore, one physiological study has shown that electrical stimulation of the chorda-lingual nerves evoked a marked salivary flow which was significantly inhibited by GABA and muscimol, an agonist of the GABA_A receptor (Shida et al., 1995). These findings indicate that the GABAergic system in salivary gland is colocalized and/or interacts

with the cholinergic system.

In this study, levels of GAD67 and GABA in acinar cells of the submandibular gland did not completely disappear with denervation. We also found that immunoreactivities for GAD67 and GABA were detected in the duct cells of the gland, but were not affected by denervation (data not shown). Our present results suggest that the GABAergic system in the submandibular gland is, at least in part, independent of the autonomic nervous system. It has been reported that GAD-like immunoreactivity in the pancreas was found in islet cells, but not in nerves (Gilon et al., 1991). Further study is necessary to elucidate the role of GAD67 in duct cells, as well as in acinar cells, and to investigate colocalization of GABA and other neurotransmitters such as acetylcholine and noradrenaline in individual fibers.

In conclusion, the results of the present study provide evidence for the expression of functional GAD67 around acinar cells in salivary gland, and suggest that the GABAergic system in this gland is closely related to the autonomic nervous system. This also suggests that the GABAergic system is involved in the inhibition of salivary secretion.

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Figure legends

Figure 1

Analysis of GAD67/65 expression in rat salivary gland. (A) Analysis of GAD67/65 mRNA expression by RT-PCR. After RT-PCR, products were electrophoresed on 2% agarose gels and visualized with ethidium bromide. (B) Comparison of relative amount of transcripts for GAD67/65 isoform mRNA by real time quantitative RT-PCR. Each value represents mean \pm S.E.M. (n=3-6). * P <0.01, significantly different from corresponding value. (C) Analysis of endogenous GAD67/65 by Western blot. Proteins of salivary gland (30 μ g) and brain (3 μ g) were electrophoresed on 7.5% SDS-polyacrylamide gels, transferred onto membranes, and treated with anti-GAD67/65 antibody. (D) Densities of GAD67/65 protein bands. GAD blots in Western analysis were estimated quantitatively with Scion imaging software. Each value represents mean \pm S.E.M. (n=5) CC; cerebral cortex, CB; cerebellum, PG; parotid gland; SLG; sublingual gland, SMG; submandibular gland.

Figure 2

Effect of denervation on weight and morphological change in rat submandibular gland. (A) Weight of submandibular gland in rats with denervation. SMG; submandibular gland. Each value represents mean \pm S.E.M. (n=4) (B) Pathological analysis of submandibular gland in rats treated with denervation. Photomicrographs show paraffin sections stained by hematoxylin/eosin (upper) and Oil red-O (lower). Arrowheads and arrows in photomicrographs show acinar cells and duct cells, respectively. Scale bars; 50 μ m.

Figure 3

Effect of denervation on GAD expression in rat submandibular gland. (A) Analysis of endogenous GAD67 by immunohistochemical study. Immunoreactivity of GAD in

submandibular gland of rats with/without denervation was determined as described in “Materials and methods”. GAD67-positive cells appear green; cell nucleus was stained with Hoechst 33342 (blue). Arrows indicate acinar cells. Scale bars; 10 μ m. (B) Quantitative analysis of change in immunoreactivity in submandibular gland. Fluorescence intensity in immunoreactivity was quantitatively analyzed using Scion imaging software. Each value represents mean \pm S.E.M. (n=4, * P <0.01) (C) Analysis of GAD67 protein expression by Western blot. Photographs show representative blots of GAD67 and β -actin. Graphs show relative densities of bands on blots estimated with Scion imaging software. Each value represents mean \pm S.E.M. (n=5, * P <0.01)

Figure 4

Effect of denervation treatment on GABA levels in rat submandibular gland. (A) Analysis of endogenous GABA by immunohistochemical study. Immunoreactivity of GABA in submandibular gland of rats with/without gabaculine was determined as described in “Materials and methods”. Gabaculine (50 mg/kg, i.p.) was injected in rat and submandibular gland was excised after 6 h. GABA-positive cells appear green; cell nucleus was stained with Hoechst 33342 (blue). Arrows indicate acinar cells. Scale bars; 10 μ m. (B) Quantitative analysis of change in immunoreactivity in submandibular gland. Fluorescence intensity in immunoreactivity was quantitatively analyzed using Scion imaging software. Each value represents mean \pm S.E.M. (n=4, * P <0.01) (C) GABA contents in submandibular gland after denervation. GABA levels were measured 14 days after denervation. Each value represents mean \pm S.E.M. (n=4, * P <0.01)

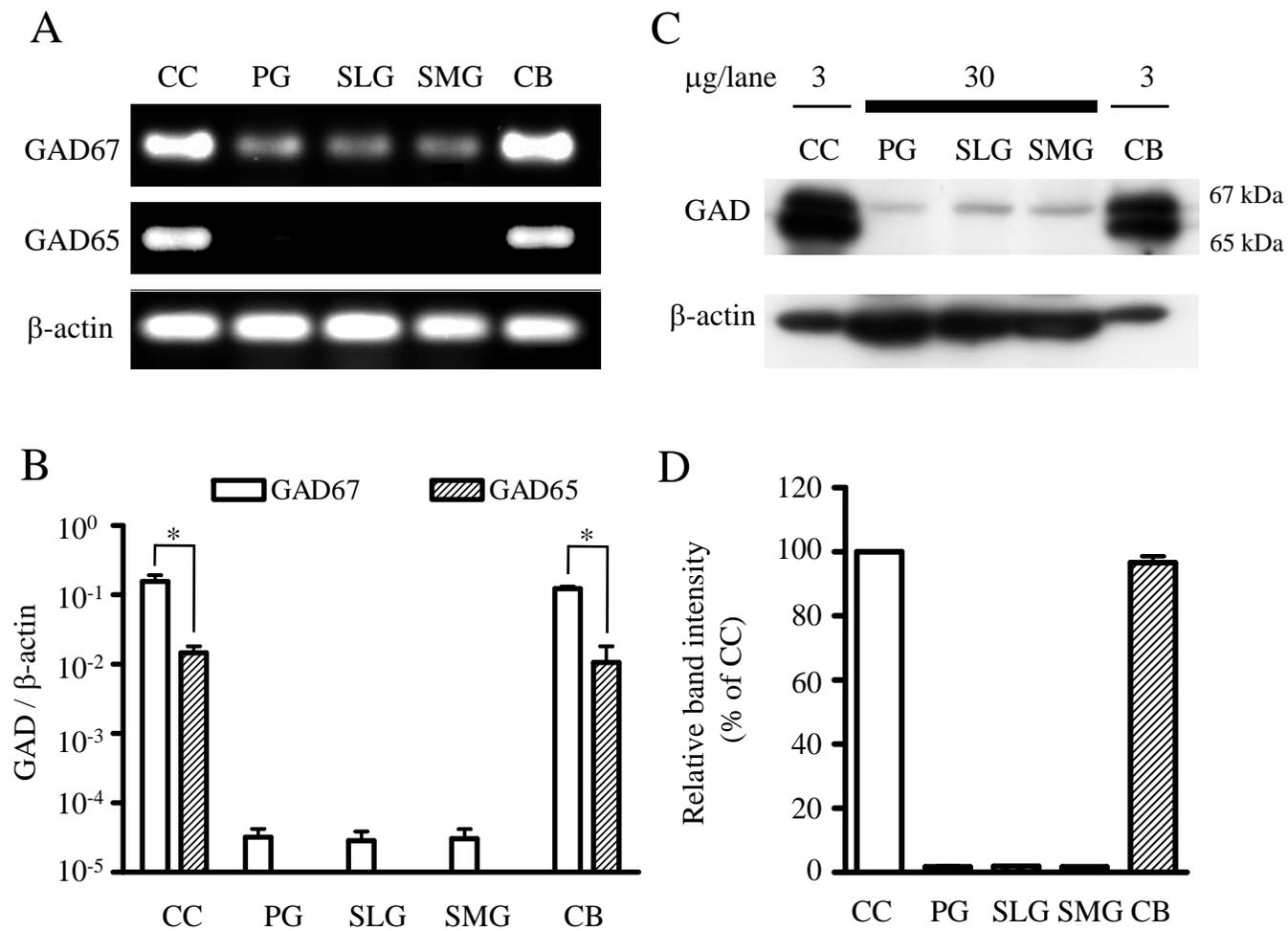


Figure 1 (Kosuge *et al.*)

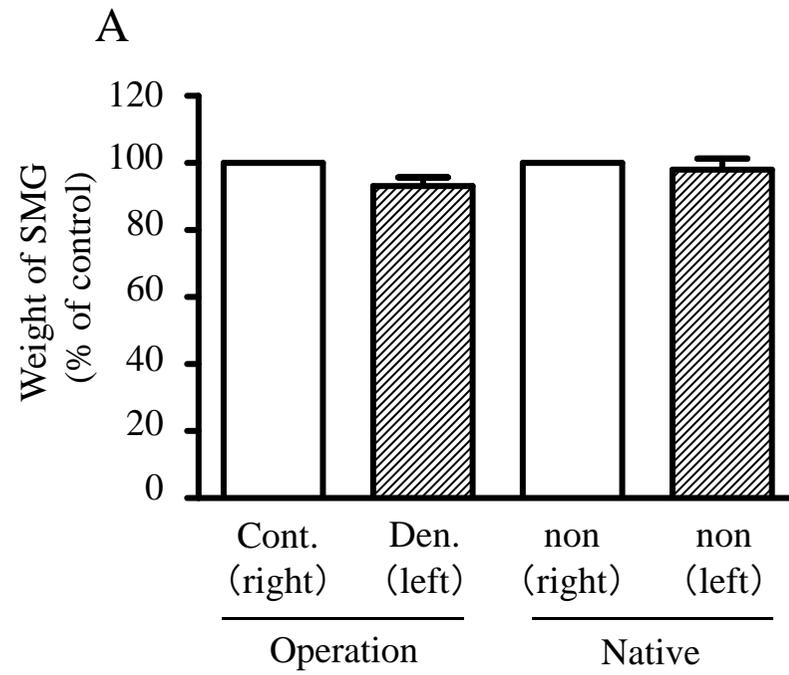


Figure 2 (Kosuge *et al.*)

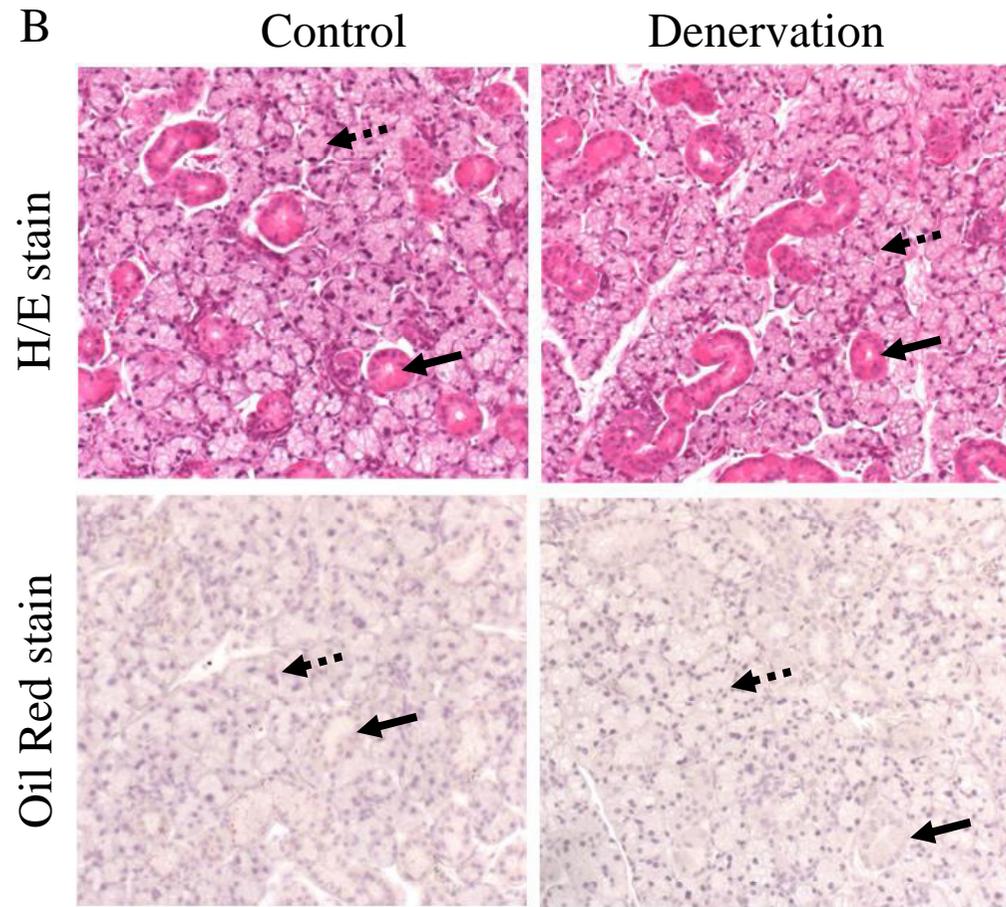
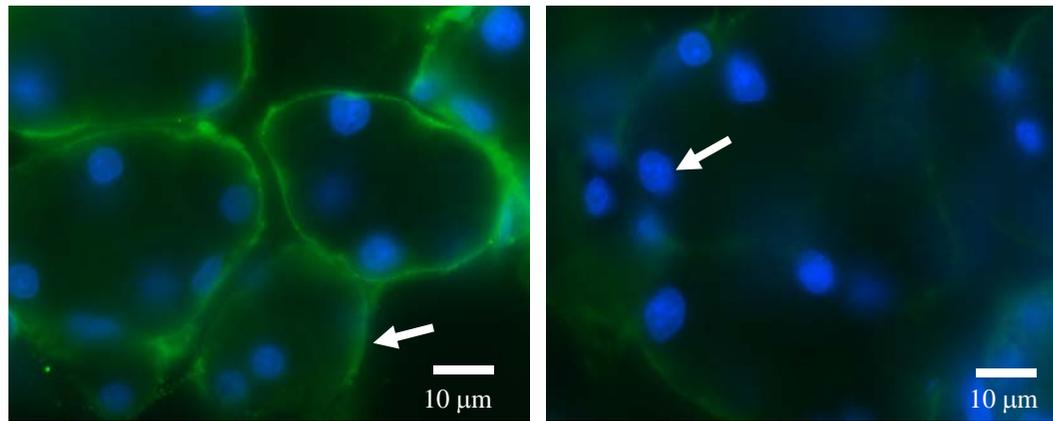


Figure 2 (Kosuge *et al.*)

A



Control

Denervation

Figure 3 (Kosuge *et al.*)

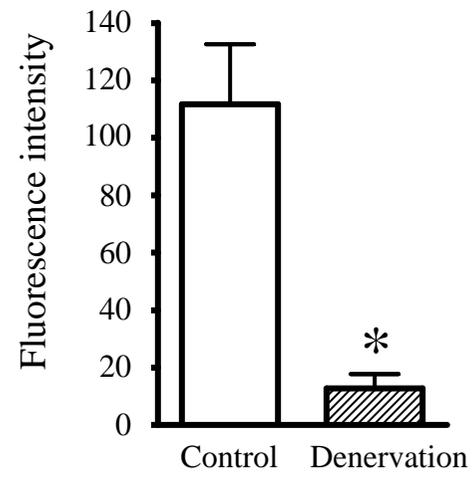
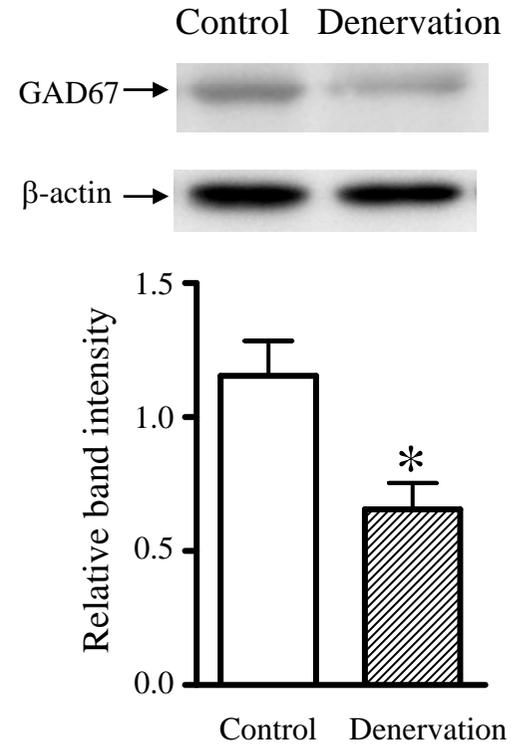
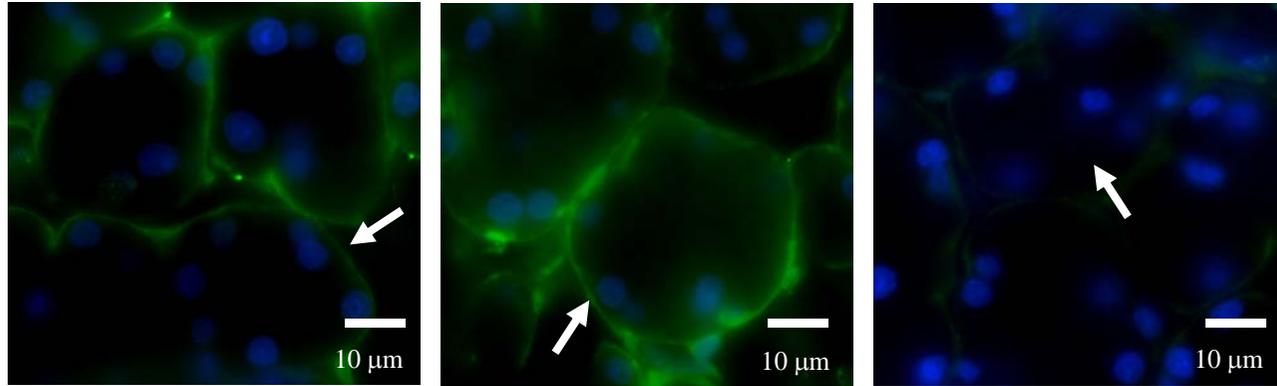
B**C**

Figure 3 (Kosuge *et al.*)

A



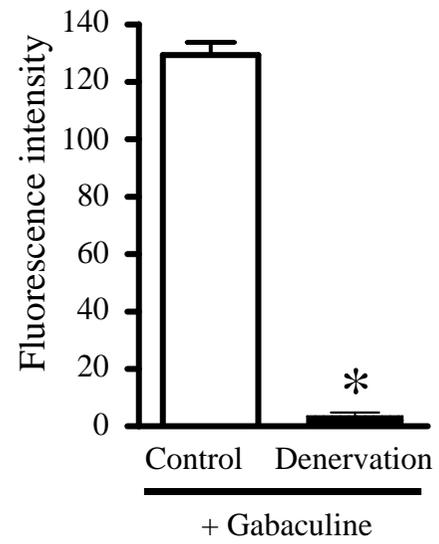
Control

+ Gabaculine

Denervation
+ Gabaculine

Figure 4 (Kosuge *et al.*)

B



C

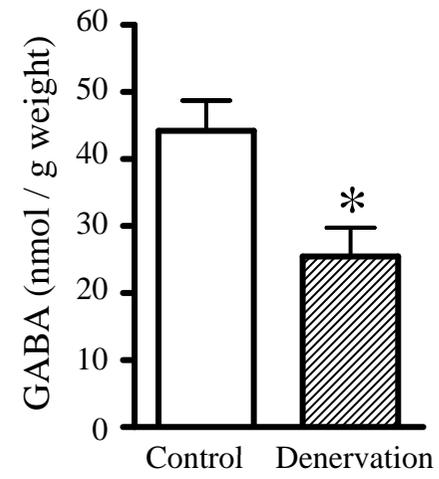


Figure 4 (Kosuge *et al.*)