

Expression Pattern of the *LacZ* Reporter in Secretogranin III Gene-trapped Mice

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Summary

Secretogranin III (SgIII) is a granin protein involved in secretory granule formation in peptide-hormone-producing endocrine cells. In this study, we analyzed the expression of the *LacZ* reporter in the *SgIII* knockout mice produced by gene trapping (*SgIII-gtKO*) for the purpose of comprehensively clarifying the expression patterns of SgIII at the cell and tissue levels. In the endocrine tissues of *SgIII-gtKO* mice, *LacZ* expression was observed in the pituitary gland, adrenal medulla, and pancreatic islets, where SgIII expression has been canonically revealed. *LacZ* expression was extensively observed in brain regions, especially in the cerebral cortex, hippocampus, hypothalamic nuclei, cerebellum, and spinal cord. In peripheral nervous tissues, *LacZ* expression was observed in the retina, optic nerve, and trigeminal ganglion. *LacZ* expression was particularly prominent in astrocytes, in addition to neurons and ependymal cells. In the cerebellum, at least four cell types expressed SgIII under basal conditions. The expression of SgIII in the glioma cell lines C6 and RGC-6 was enhanced by excitatory glutamate treatment. It also became clear that the expression level of SgIII varied among neuron and astrocyte subtypes. These results suggest that SgIII is involved in glial cell function, in addition to neuroendocrine functions, in the nervous system. (J Histochem Cytochem 69: 229–243, 2021)

Keywords

Bergmann glia, cerebrospinal fluid, gene knockout, glial response, gliocrine, neuroendocrine, X-gal staining

Introduction

Secretogranin III (SgIII) is a member of the granin protein family that is involved in secretory granule formation in peptide-hormone-producing endocrine cells. The granin family consists of seven types of acidic secretory proteins: chromogranin A (CgA), chromogranin B (CgB), secretogranin II (SgII), and SgIII–VI.^{1–4} Some of the members of the granin family, such as CgA, CgB, SgII, and SgIII, may participate in the aggregation/condensation process of peptide hormones during the biosynthesis of secretory granules (SGs).⁵ In this process, SgIII has been suggested to play a role as a bridge molecule between the peptide hormone/CgA core aggregates and the cholesterol-rich microdomains of SG membranes.^{6,7} Furthermore, SgIII has been found to bind to membrane-associated carboxypeptidase E (CPE), a prohormone-processing (end-modifying) enzyme that has been proposed as a candidate molecule for the

sorting receptor of prohormones.⁸ These findings suggest that the cooperative formation of a sorting platform consisting of SgIII, CgA, and CPE at the trans-Golgi network is crucial for SG formation.^{6,8,9} SgIII was originally identified as a neuron-specific gene *1B1075* product in mice.^{10–12} Immunohistochemical and in situ hybridization analyses have shown the expression of SgIII in the nervous and endocrine tissues of various organisms.^{6,11,13–17} However, it does not have completely

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understood the expression of SgIII of more detailed cell or tissue structure.

We have established a strain of SgIII gene-deficient mice via the gene trap method (*SgIII-gtKO*) and conducted analyses at the individual level. We found that *SgIII-gtKO* mice exhibited no overt abnormalities under ordinary rearing conditions. However, these animals were highly vulnerable to various loading conditions compared with normal wild-type control (*WT*) mice. For example, they showed marked obesity and impaired glucose tolerance when fed a high-fat/high-sucrose diet. These phenotypes were associated with increased proinsulin accumulation, accompanied by hypertrophy of pancreatic islets and decreased active insulin secretion. Furthermore, the *SgIII-gtKO* mice exhibited a small elevation of plasma adrenocorticotrophic hormone (ACTH) content from the pituitary gland after chronic restraint stress. Thus, the lack of *SgIII* caused maladaptation of endocrine cells to inadequate diet and stress by impairing the proteolytic conversion of prohormones in SGs.¹⁸

The *SgIII-gtKO* strain was produced using gene trapping, in which the gene trap vector is inserted into intron 11 of the *SgIII* genome on chromosome 9, followed by the induction of β -Geo (a fusion gene of the *LacZ reporter* and neomycin-resistance genes) expression by the authentic *SgIII promoter*. Therefore, this model has the advantage of being able to visualize the *SgIII* expression sites with high sensitivity, which renders it an effective tool for detailed cell/tissue expression analyses. In the central nervous system (CNS), the *SgIII* transcripts and protein have been found widely in brain regions and seem to be predominantly expressed in neuronal elements associated with neuroendocrine function and monoaminergic neurotransmission.^{11,17,19} More interestingly, it has been reported that the expression of SgIII in astrocytes is involved in the astrocytic secretory pathway and is regulated by the glial activation state.¹⁶ Here, we studied the expression of SgIII extensively in the nervous tissues using the *LacZ reporter* and identified the subtypes of SgIII-expressing neurons and astrocytes. We demonstrated that *LacZ* expression was observed extensively in the central and peripheral nervous tissues and was pronounced in glial cells. In addition, using rat glial cell lines, we clarified the relationship between SgIII expression and the activated glial response induced by glutamate stimulation.

Materials and Methods

Gene Trapping and *SgIII-gtKO* Mice

The gene trapping used in this study has been published elsewhere.^{20,21} The structure of the gene-trapping

cassette, its genomic insertion site, and the expression of a fusion gene of the *LacZ* reporter and neomycin-resistance genes (β -Geo) in *SgIII-gtKO* mice (B6;129S-Scg3^{tm1Lex} strain; The Jackson Laboratory, Bar Harbor, ME) are shown in Fig. 1. The *SgIII-gtKO* and *WT* mice were generated in the genetic background of the C57BL/6J strain by the intercrossing of heterozygous mice. The genotypes of the offspring were determined by amplifying the *SgIII* alleles and the gene-trapping cassette by polymerase chain reaction using the appropriate primers, as previously reported.¹⁸ The mice were maintained on a 12-hr light/dark cycle and given free access to tap water and standard chow (CRF-1; Oriental Yeast, Osaka, Japan). All animal experiments were performed in compliance with relevant laws and the guidelines of the Care and Use of Laboratory Animals of the Research Council of Akita Prefectural University (approval number 17-03 and 18-02).

LacZ Staining

For *LacZ* staining, tissue processing was performed as described previously.^{22,23} Five-month-old male mice ($n=5$) were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (0.1 mg/g body weight) and fixed with 50 ml of ice-cold 3.8% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) via cardiac perfusion for 10 min after washing out the blood with physiological saline perfusion for 3 min. The brain, spinal cord, and peripheral nervous and endocrine tissues were removed and postfixed in 1.9% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4C overnight. The isolated islets (as described below) were also fixed by immersion in 3.8% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4C overnight. Fixed brains were embedded in 2% agarose in 0.05 M sodium phosphate buffer (pH 7.4) and sliced coronally on a Microslicer DTK-1000 instrument (Dosaka EM; Kyoto, Japan) at a thickness of 400 μ m. Slices were rinsed in 0.05 M sodium phosphate buffer (pH 8.0) 3 \times and reacted in 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) solution (0.1 M sodium phosphate buffer [pH 8.0], 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂, and 1 mg/ml X-gal) for 12 hr at 37C with gentle shaking, as described elsewhere.²⁴ After the *LacZ* staining, tissues were postfixed in 3.8% formaldehyde in 0.1 M sodium phosphate buffer (pH 8.0). Images were acquired using an MZ75 stereomicroscope (Leica Microsystems; Wetzlar, Germany) equipped with a DP72 CCD camera and a KL1600 LED lighting apparatus (Olympus; Tokyo, Japan). Part of the *LacZ*-stained tissue was embedded in paraffin for histological analysis.

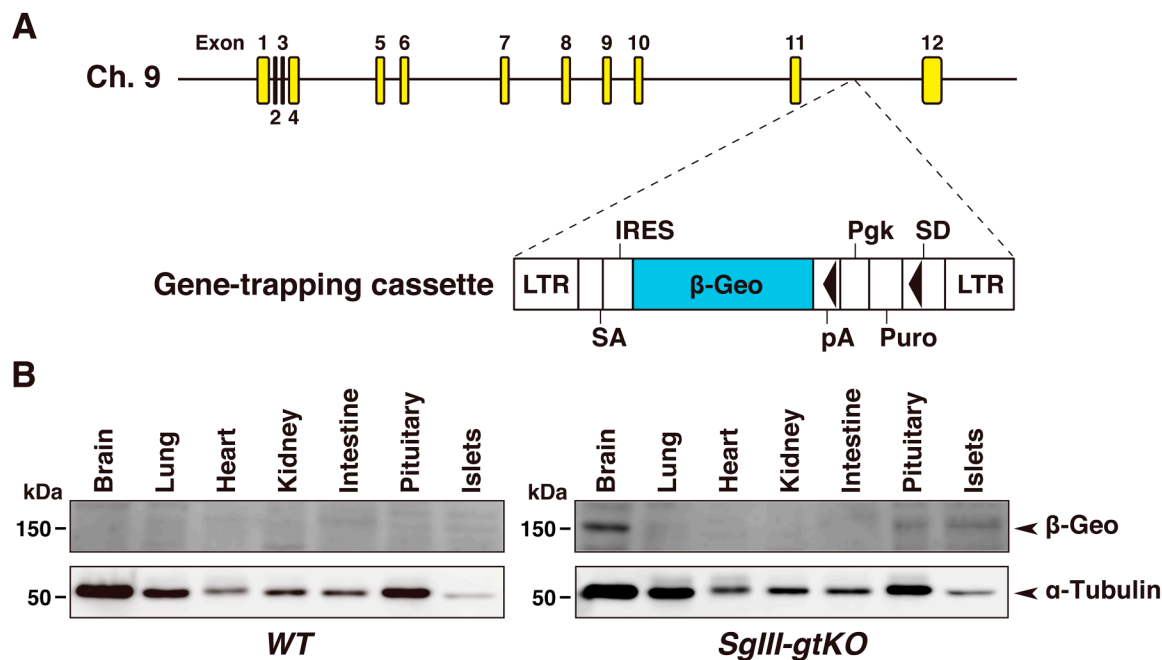


Figure 1. Structure of the *SgIII* gene trap vector and genomic insertion. (A) The gene trap vector was inserted on mouse chromosome 9, between exons 11 and 12 of the *SgIII* genome.¹⁸ The gene-trapping cassette contains a splice-acceptor site (SA) positioned at the 5' end of a promoterless reporter region, β -geo (a fusion of neomycin phosphotransferase and β -galactosidase), allowing the vector to be spliced to the endogenous gene with an internal ribosomal re-entry site (IRES) between the SA site and the reporter gene sequence. The puromycin resistance gene (*Puro*) is also expressed when the gene trap vector is inserted into an intron under the control of a constitutive phosphoglycerate kinase gene (*Pgk*) promoter, which is thought to trap a gene to be transcriptionally silent in mouse embryonic stem cells. As a result, the *LacZ*-reporter gene can be translated even when it is not fused to the trapped gene.²¹ The black triangles in the cassette indicate the *loxP* sites. Abbreviations: LTR, retroviral long terminal repeat; SD, splice donor site; *SgIII*, secretogranin III. (B) Expression of β -Geo detected by immunoblotting using an anti- β -galactosidase antibody (upper panels). Equal amounts of protein were extracted from each tissue (20 μ g) of WT or *SgIII*-gtKO mice. Loading controls were provided by α -tubulin immunoblotting (lower panels).

Isolation of Pancreatic Islets

Pancreatic islets were isolated by collagenase digestion methods from 5-month-old male mice ($n=4$) as described previously.²⁵ Briefly, 6 ml of collagenase solution (500 U/ml) (C-7657, type XI; Sigma-Aldrich, Inc. St. Louis, MO) was injected from the duodenal papilla of the pancreatic duct, and the pancreas was excised from the abdomen and digested at 37°C for 30 min. After digestion, the tissue was dispersed by gentle pipetting and islets were isolated from exocrine tissues. The islets were handpicked under a stereomicroscope and used for *LacZ* staining and immunoblotting.

Primary Antibodies

The rabbit polyclonal anti-SgIII antibody raised against rat SgIII peptides 373–471⁷ was used for immunohistochemistry and immunoblotting (1:2000 dilution). The mouse monoclonal anti- β -galactosidase antibody (1:2000 dilution, Z3781; Promega, Madison, WI) was

used for immunoblotting to analyze the tissue expression of *LacZ*. The mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibodies (1:200 dilution, GF5, ab10062; Abcam, Cambridge, UK and 1:60 dilution, GF12.24; Progen Biotechnik GmbH, Heidelberg, Germany) and rabbit polyclonal anti-glutamine synthetase (GS) antibody (1:20,000 dilution, ab49873; Abcam) were used for immunohistochemistry and immunoblotting. The mouse monoclonal anti- α -tubulin antibody (1:3000 dilution, T5168; Sigma-Aldrich, Inc.), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:500 dilution, ab9484; Abcam), and anti- β -actin antibody (1:5000 dilution, A5316; Sigma-Aldrich, Inc.) were used for loading control in the immunoblot analysis.

Immunohistochemistry

Tissues of 4- to 6-month-old male mice ($n=5$) were excised and fixed in Bouin's fixative for 3 days. Tissue blocks were then dehydrated and embedded in

paraffin. Five- μ m-thick sections were mounted on silane-coated glass slides (Muto Pure Chemicals; Tokyo, Japan) and processed for immunohistochemistry as described previously.¹⁷ For SgIII immunohistochemistry, deparaffinized sections were immersed in 0.01 M sodium citrate buffer (pH 6.0) and processed using microwave antigen retrieval. Subsequently, the sections were treated with Dako REAL Peroxidase-Blocking Solution (Dako; Glostrup, Denmark) and blocked with 5% normal goat serum (Vector Laboratories; Burlingame, CA) in PBS. After incubation with primary antibodies overnight at 4C, the sections were rinsed in PBS and incubated with secondary antibodies using EnVision Dual Link System horseradish peroxidase (HRP)-labeled polymer reagent (Dako). The sections were then visualized using a peroxidase substrate kit (SK-4100; Vector Laboratories) and counterstained with Mayer's hematoxylin. The control experiment in the immunohistochemistry followed the literature which is describing its guidelines.²⁶ As a negative control, sections were incubated with normal rabbit or mouse IgG (Santa Cruz; sc-2027 or sc-2025, 1 μ g/mL) instead of the primary antibodies; we confirmed that there were no significant immunosignals in this case. Images were acquired using a BX51 microscope equipped with a DP72 CCD camera and digitized with the DP2-BSW software (Olympus).

Dual Staining of LacZ Expression and Immunohistochemistry

LacZ-stained tissues were embedded in 0.4% Agarose L (317-01182; Nippon Gene Co. Ltd., Tokyo, Japan) and 1% gelatin (16605-42; Nacalai Tesque, Kyoto, Japan) in 0.05 M sodium phosphate buffer (pH 7.4) and refixed in Bouin's fixative for 24 hr. The tissue blocks were dehydrated and embedded in paraffin. Subsequently, deparaffinized sections were processed by antigen retrieval, reacted with primary antibodies, and incubated with secondary antibodies using the HRP-labeled polymer technique, as mentioned above.

Immunoblotting

For immunoblot analysis, tissue samples from 5-month-old male mice ($n=6$) were collected, frozen on dry ice, and stored at -80°C until use. Frozen tissues were thawed and homogenized with lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 2.5 mM MgCl_2 , 1 mM EGTA, 1.0% Triton X-100, and a protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Mannheim, Germany). The homogenates were spun at $20,000 \times g$ for 10 min, the supernatants were collected, and

the protein concentration was determined using a Coomassie brilliant blue protein assay reagent (Nacalai Tesque Inc.), as described previously.¹⁷ Each protein extract was normalized for total protein content (5–40 μ g), subjected to SDS-PAGE, and transferred to an Immobilon-P transfer membrane (Millipore; Billerica, MA). After blocking with 5% skimmed milk in Tris-buffered saline (pH7.4) supplemented with 0.05% Tween 20 for 1 hr, the membrane was incubated with primary antibodies. The peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratory; West Grove, PA), anti-rabbit IgG (cat. no. 111-035-144), and anti-mouse IgG (cat. no. 111-035-146) were diluted (1:5000) and used. Signal detection was performed using enhanced chemiluminescent Western blotting detection reagents (PRN2106; GE Healthcare Bio-Sciences KK, Tokyo, Japan).

Glial Cell Lines and Cell Culture

The rat-glioma-derived C6 (resource no. RCB2854) and RGC-6 (resource no. RCB2783) cell lines were provided by RIKEN BRC through the National BioResource Project of the MEXT, Japan. The C6 cell line was maintained in RPMI1640 (11875-093; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; S1780-500, Japan Bioserum, Tokyo, Japan), and the RGC-6 cell line was maintained in MEM (M4655; Sigma-Aldrich Inc.) supplemented with 10% FBS. These cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2 .

To evaluate astrocytic SgIII expression, activation of glioma cells was performed as follows. C6 and RGC-6 glioma cell lines grown in six-well plates to 80% confluency were stimulated with L-glutamic acid (13012-92; Nacalai Tesque Inc., concentrations of 1 or 2 mM) and cultured for 24 hr.²⁷ After stimulation, cells were lysed and solubilized in 20 mM HEPES (pH 7.4) containing 0.1 M NaCl, 1 mM EDTA, 1% Triton X-100, 0.4 mM phenylmethanesulfonyl fluoride, and a protease inhibitor cocktail (Nacalai Tesque Inc.). The cell lysates were centrifuged at $11,000 \times g$ for 10 min at 4°C to obtain protein sample solutions. Aliquots of the soluble extracts were assessed by SDS-PAGE followed by immunoblotting. The quantities of SgIII were estimated via densitometry of the immunoblot bands with an appropriate molecular weight (70 kDa) using the LAS4000 (General Electric Company; Fairfield, CT) software.

Statistical Analysis

Statistical significance was evaluated using Student's *t*-tests, and $p < 0.05$ was considered significant.

Statistical significance is indicated in each figure legend as follows: * $p < 0.05$, ** $p < 0.01$.

Results

Expression of SgIII/LacZ in Canonical Endocrine Tissues

The gene trap vector was inserted between exons 11 and 12 of the *SgIII* genome on chromosome 9 (Fig. 1A).¹⁸ We first examined the *SgIII/LacZ* expression in *SgIII-gtKO* mice by immunoblotting using anti-SgIII and anti- β -galactosidase antibodies in the canonical endocrine tissues, where the SgIII expression had previously been established.^{6,13–15,17} The expression of β -galactosidase was detected in the brain, pituitary gland, and pancreatic islets in *SgIII-gtKO*, but not in *WT* mice (Fig. 1B). In *WT* mice, positive signals for SgIII were detected in endocrine tissues, whereas no significant signal was detected in those of *SgIII-gtKO* animals (Fig. 2A). To determine whether the gene inserted in the *SgIII* genomic locus is expressed similarly as the endogenous *SgIII*, we then examined the consistency between the *SgIII* and *LacZ* expression by immunohistochemistry and histochemical X-gal staining. In immunohistochemical staining, a moderate-to-strong positive immunoreaction was observed in the glandular (anterior and intermediate lobes) and neural (posterior lobe) pituitary, adrenal medulla, and pancreatic islets in *WT* mice, whereas no immunoreaction was detected in *SgIII-gtKO* mice (Fig. 2B). Equivalently, *LacZ* expression in these tissues was detected in *SgIII-gtKO* mice (Fig. 3). The expression pattern in these endocrine tissues correlated well with that of SgIII determined by immunohistochemistry. Thus, it was revealed that the gene-trapping cassettes were well controlled by endogenous *SgIII*-promoter activity in *SgIII-gtKO* mice, and that this mouse strain is useful for the sensitive examination of *SgIII* expression.

LacZ Expression in the Brain and Spinal Cord

To determine the *LacZ* expression in the CNS, we then conducted a comprehensive analysis using coronal tissue slices from *SgIII-gtKO* mice. The β -galactosidase activity was detected in various brain regions and the spinal cord, including the olfactory bulb, cerebral cortex, caudate putamen, hypothalamus, hippocampus, thalamus, amygdala, midbrain, cerebellum, pons, medulla oblongata, and gray matter of the spinal cord of *SgIII-gtKO* mice (Fig. 4A–E and K–O), whereas no β -galactosidase activity was detected in control *WT* mice (Fig. 4F–J and P–T). *LacZ* expression was strong in the cerebral cortex, hippocampus, and molecular

layer of the cerebellum and moderate in the caudate putamen, hypothalamus, amygdala, midbrain, medulla oblongata, and dorsal (posterior) horn of the spinal cord.

In the slices of cerebral cortex and hippocampus, the cortical layers II and III and the dentate gyrus (DG) exhibited a stronger expression of *LacZ* (Fig. 5A and B). A moderate β -galactosidase activity was detected in other cortical areas and hippocampal cornu ammonis (CA) fields. The paraventricular nuclei (Fig. 5C and D), dorsomedial nuclei, and arcuate nuclei (Fig. 5E and F) of the rostral and intermediate hypothalamus showed a moderate expression of *LacZ*. Moreover, the molecular layer and piriform neuron layer of the cerebellar cortex showed a stronger expression of *LacZ* (Fig. 5G and H).

In the section-level analysis, GFAP-positive astrocytes in the hippocampal DG and cerebral cortex were the main cellular elements that expressed *LacZ* (Fig. 6A–D). In the hippocampus, CA pyramidal neurons and DG granular cells did not exhibit β -galactosidase activity (Fig. 6B). Conversely, some of the pyramidal neurons of the cerebral cortex expressed *LacZ* (Fig. 6C). The perivascular structure showed an overlapping of the *LacZ* blue staining and GFAP-positive perivascular astrocytes (Fig. 6D). In the cerebellar cortex, Purkinje cells, Bergmann glia (which are a GFAP-positive cerebellar-specific subtype of astrocytes), and a limited number of cells located in the granular layer near the piriform neuron layer expressed *LacZ* (Fig. 6E–G). In addition to these cells, *LacZ* expression was observed in GFAP-positive astrocytes localized in the medulla oblongata (data not shown). In the lateral ventricle, ependymal cells expressed *LacZ* (Fig. 6H).

LacZ Expression in the Peripheral Nervous Tissues

To assess the *LacZ* expression in the peripheral nervous tissues, we analyzed the retina, optic nerve, and trigeminal ganglion. In the retina, *LacZ* expression was detected in the ganglionic cell layer, inner granular layer, and outer granular layer (Fig. 7A–C). A weak expression of *LacZ* was observed in the optic nerves (Fig. 7D), and expression in glial cells was inferred from transverse section images (Fig. 7E). In the trigeminal ganglion, a moderate β -galactosidase activity was detected in the assumed ganglion cells and ganglionic glial cells (Fig. 7F). No β -galactosidase activity was detected in these tissues of *WT* mice (Fig. 7G–I). To identify the *LacZ*-expressing cellular elements, we then performed a section-level analysis. In the optic nerve, GFAP-positive astrocytes expressed *LacZ* (Fig. 7J and K). In the trigeminal ganglion, *LacZ* expression

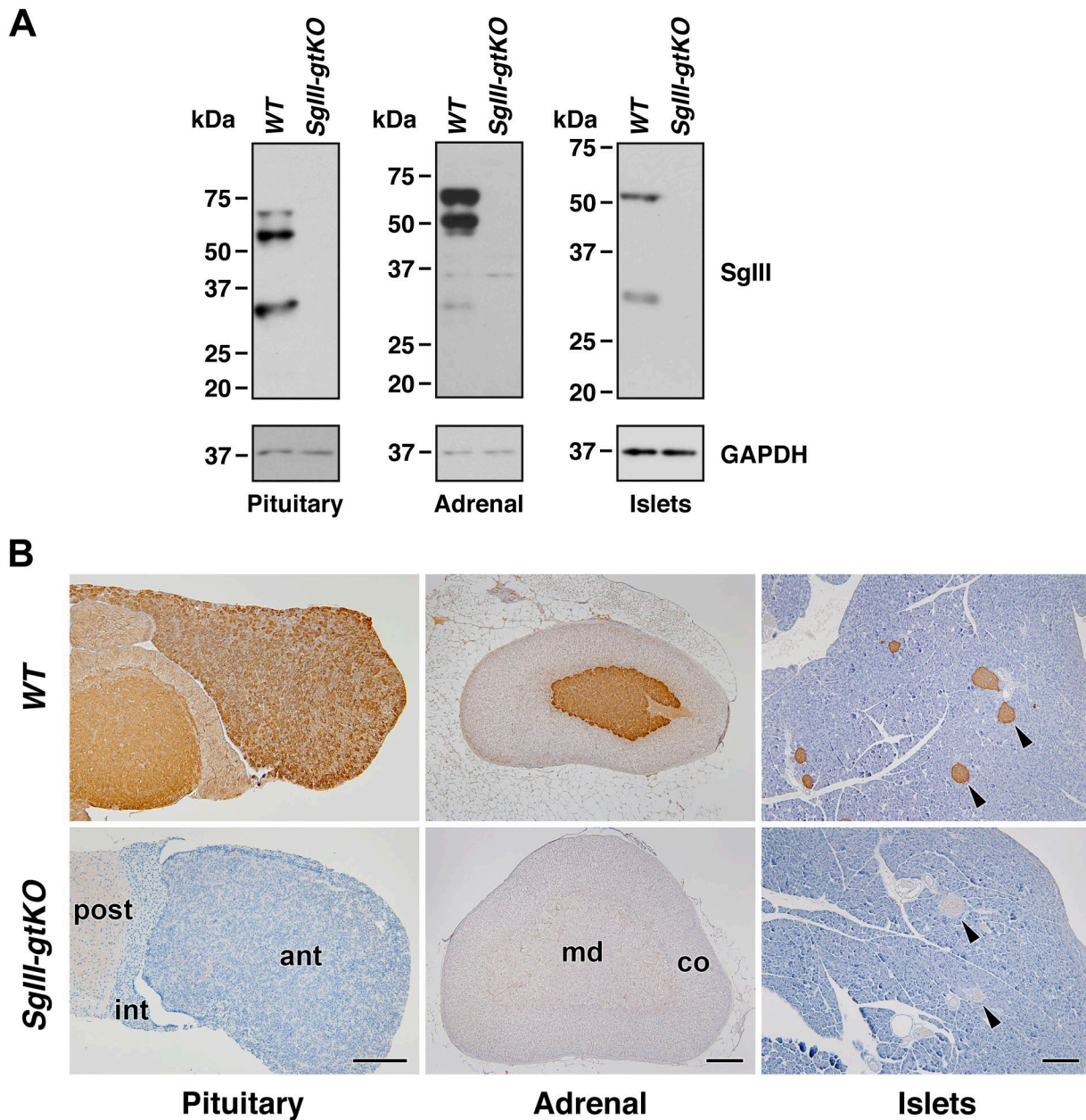


Figure 2. Expression of SgIII in canonical endocrine tissues. (A) Immunoblot analysis. Equal amounts of the protein extracts from the pituitary glands (10 μ g), adrenal glands (10 μ g), and pancreatic islets (5 μ g) of WT or *SgIII-gtKO* mice were used for SDS-PAGE, followed by immunoblotting using anti-SgIII and anti-GAPDH antibodies. (B) Immunohistochemical analysis. Paraffin sections of pituitary glands, adrenal glands and pancreases from *SgIII-gtKO* and WT mice were immunostained with anti-SgIII antibody. Arrowheads indicate pancreatic islets. Sections were counterstained with hematoxylin. Scale, 50 μ m. Abbreviations: ant, anterior pituitary lobe; int, intermediate pituitary lobe; post, posterior pituitary lobe; co, adrenal cortex; md, adrenal medulla; SgIII, secretogranin III; WT, wild-type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

was observed in some ganglion cells and surrounding satellite cells (Fig. 7L and M).

Expression of SgIII in the Cerebellum

Although there are reports that SgIII is expressed at several sites in the CNS,^{11,16,19} knowledge about

cerebellar SgIII expression has not been fully obtained. Therefore, we further analyzed the cerebellar expression of SgIII in WT and *SgIII-gtKO* mice. An immunoblotting analysis revealed the expression of three fragmented bands of SgIII in the cerebellum of WT mice (Fig. 8A), suggesting a similar proteolytic processing from the SgIII precursor as that observed in

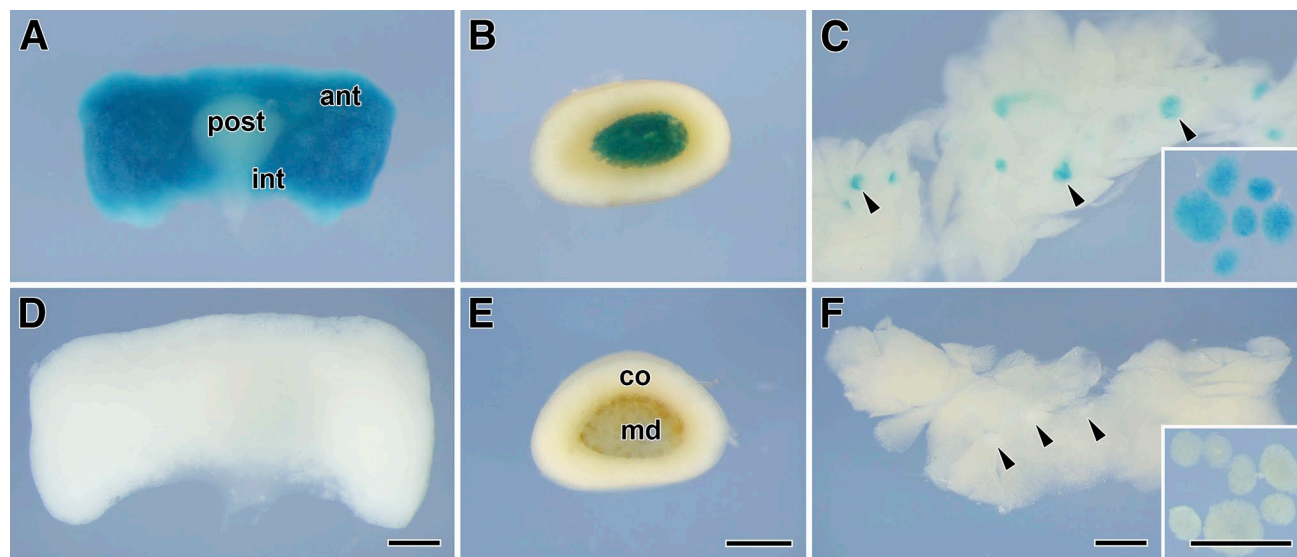


Figure 3. *LacZ* expression in endocrine tissues. Whole-mount *LacZ* staining of the pituitary glands (A, D), adrenal glands (B, E), and pancreases (C, F) from *SgIII-gtKO* (A–C) and WT (D–F) mice. Arrowheads in (C) and (F) indicate the pancreatic islets, and the inset panels show the isolated islets. Scale, 0.5 mm. Abbreviations: ant, anterior pituitary lobe; int, intermediate pituitary lobe; post, posterior pituitary lobe; co, adrenal cortex; md, adrenal medulla; *SgIII*, secretogranin III; WT, wild-type.

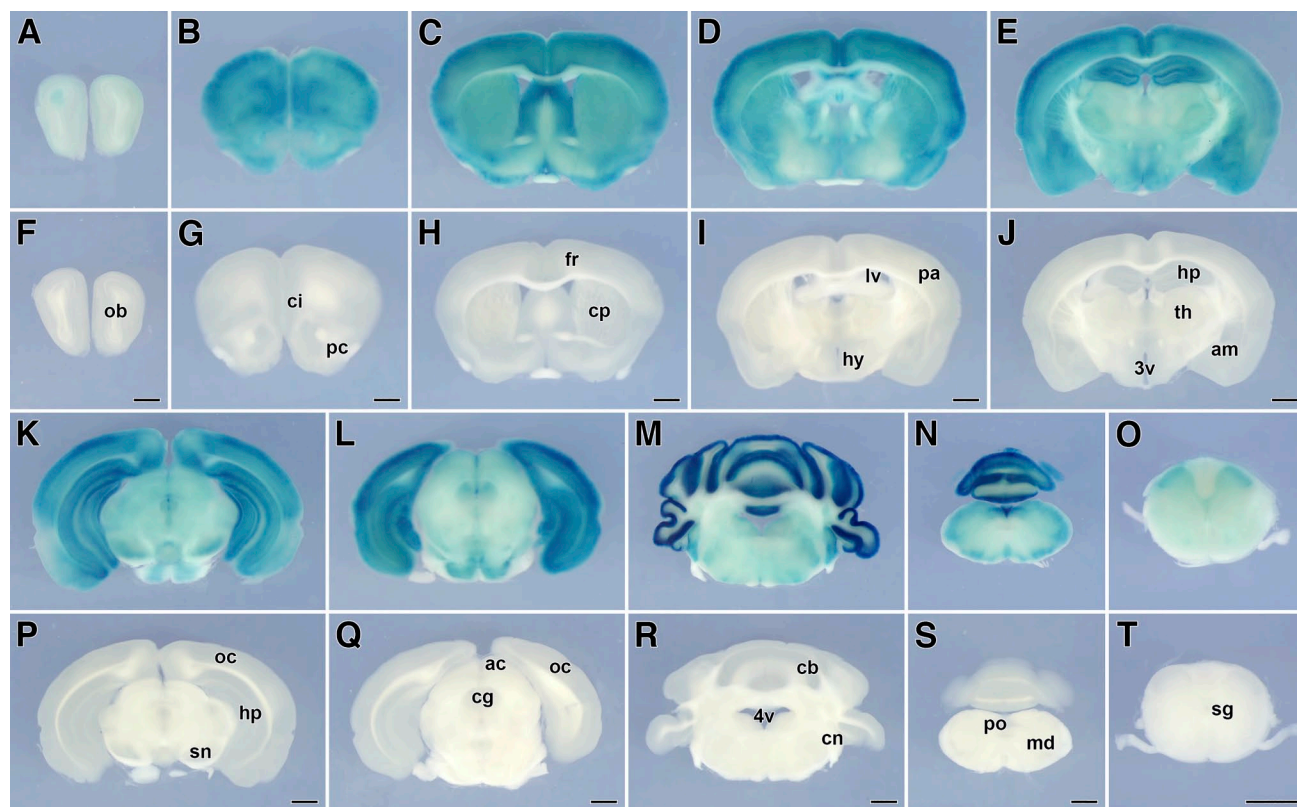


Figure 4. *LacZ* expression in slice preparations of the brain and spinal cord. The panels from (A) to (E) and (K) to (O) of *SgIII-gtKO* or (F) to (J) and (P) to (T) of WT mice are arranged from the rostral to the caudal. Scale, 1 mm. Abbreviations: ac, anterior colliculi; am, amygdaloid nuclei; cb, cerebellar cortex; cg, central gray; ci, cingulate cortex; cn, cochlear nuclei; cp, caudate-putamen; fr, frontal cortex; hp, hippocampus; hy, hypothalamus; lv, lateral ventricle; md, medulla oblongata; ob, olfactory bulb; oc, occipital cortex; pa, parietal cortex; pc, piriform cortex; sn, substantia nigra; sg, gray matter of the cervical spinal cord; th, thalamus; 3v, third ventricle; 4v, fourth ventricle; *SgIII*, secretogranin III; WT, wild-type.

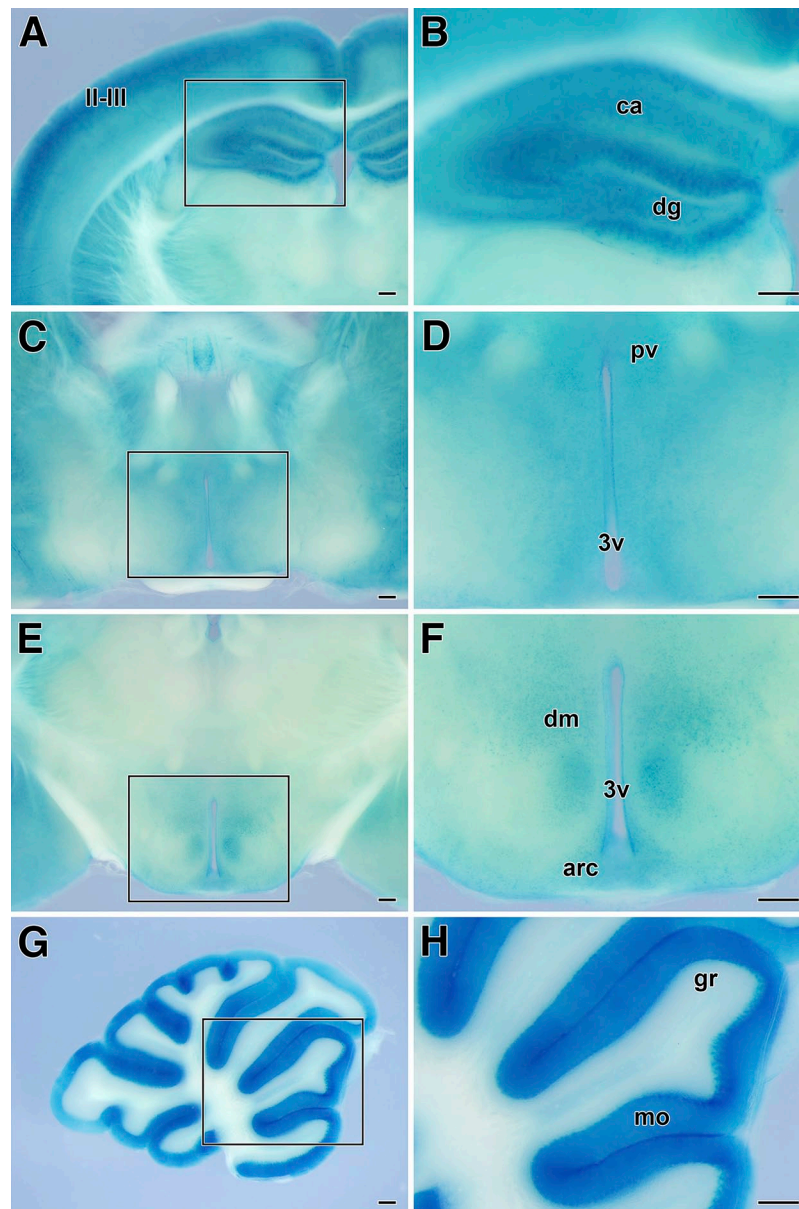


Figure 5. *LacZ* expression in brain tissues of *SgIII-gtKO* mice. Strong *LacZ* expression was observed in the slices of the cerebral cortex and hippocampus (A), rostral hypothalamus (C), intermediate hypothalamus (E), and cerebellum (G). The panels on the right (B, D, F, and H) are enlarged images of the rectangles enclosed by the line in the images on the left. Scale, 250 μ m. Abbreviations: arc, arcuate nuclei; ca, cornu ammonis; dg, dentate gyrus; dm, dorsomedial hypothalamic nuclei; gr, granular layer of the cerebellar cortex; mo, molecular layer of the cerebellar cortex; pv, paraventricular hypothalamic nuclei; II/III, layers II and III of the cerebral cortex; 3v, third ventricle; *SgIII*, secretogranin III.

the adrenal gland, compared with other endocrine tissues (Fig. 2A).

We then analyzed the cellular elements that express *SgIII* in the cerebellum by immunohistochemistry (Fig. 8B). Purkinje cells and Bergmann glia in the piriform neuron layer and Golgi-like cells showing negative immunoreactivity of GS and localizing in the granular layer expressed *SgIII* strongly. The molecular layer, basket-like cells, and neuropil around the granule cells

showed a moderate level of *SgIII* expression. Slightly stronger expression in the granular layer was observed in the assumed cerebellar glomerulus, an intertwined mass of nerve fibers consisting of granule cell dendrites and Golgi cell axon terminals surrounding mossy fiber terminals. Conversely, GS immunoreactivity-positive gray matter astrocytes in the granular layer and fibrous astrocytes located in the white matter exhibited negligible *SgIII* expression.

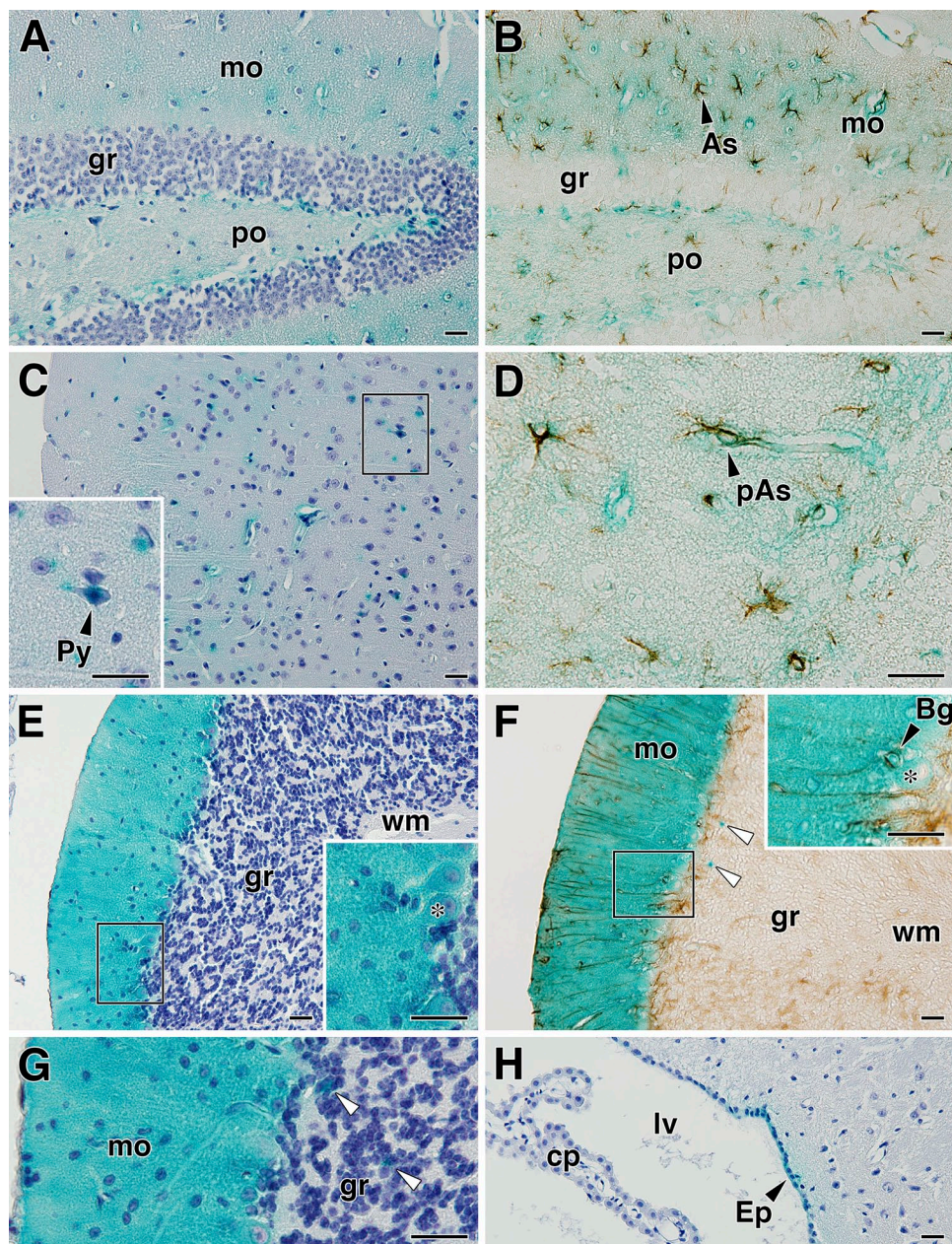


Figure 6. *LacZ* expression in cellular elements in brain tissues of *SgIII-gtKO* mice. After *LacZ* staining, paraffin sections were prepared and counterstained with hematoxylin (A, C, E, G, and H) or reacted with anti-GFAP antibodies (B, D, and F). The panels show the hippocampal DG (A and B), cerebral cortex (C and D), cerebellum (E–G), and lateral ventricle and ependyma (H). The insets in (C), (E), and (F) are enlarged images of the rectangles enclosed by the line. The inset in (C) shows large pyramidal neurons in layer V, and that in (F) shows Purkinje cells and GFAP-immunolabeled Bergmann glia and their radial processes. The asterisks on the insets in (E) and (F) indicate Purkinje cells. The open arrowheads in (F) and (G) indicate *LacZ*-expression-positive cells in the granular layer. Scale, 20 μ m. Abbreviations: Bg, Bergmann glia; cp, choroid plexus; Ep, ependymal cell; gr, granular layer; lv, lateral ventricle; mo, molecular layer; pAs, perivascular astrocyte; po, polymorphic layer; Py, pyramidal neuron; wm, white matter; *SgIII*, secretogranin III; GFAP, glial fibrillary acidic protein; DG, dentate gyrus.

Glial Expression and Reactive Induction of *SgIII*

SgIII expression in glial cells, mostly interpreted as astrocytes in the CNS, has been reported.¹⁶ Our *LacZ*-reporter-based analysis of *SgIII* expression revealed

that cortical and hippocampal astrocytes and cerebellar Bergmann glia constitutively expressed *SgIII*. In contrast, it was extremely difficult to detect *SgIII* expression in cerebellar gray matter astrocytes in the granular layer and fibrous astrocytes in the white matter. Among

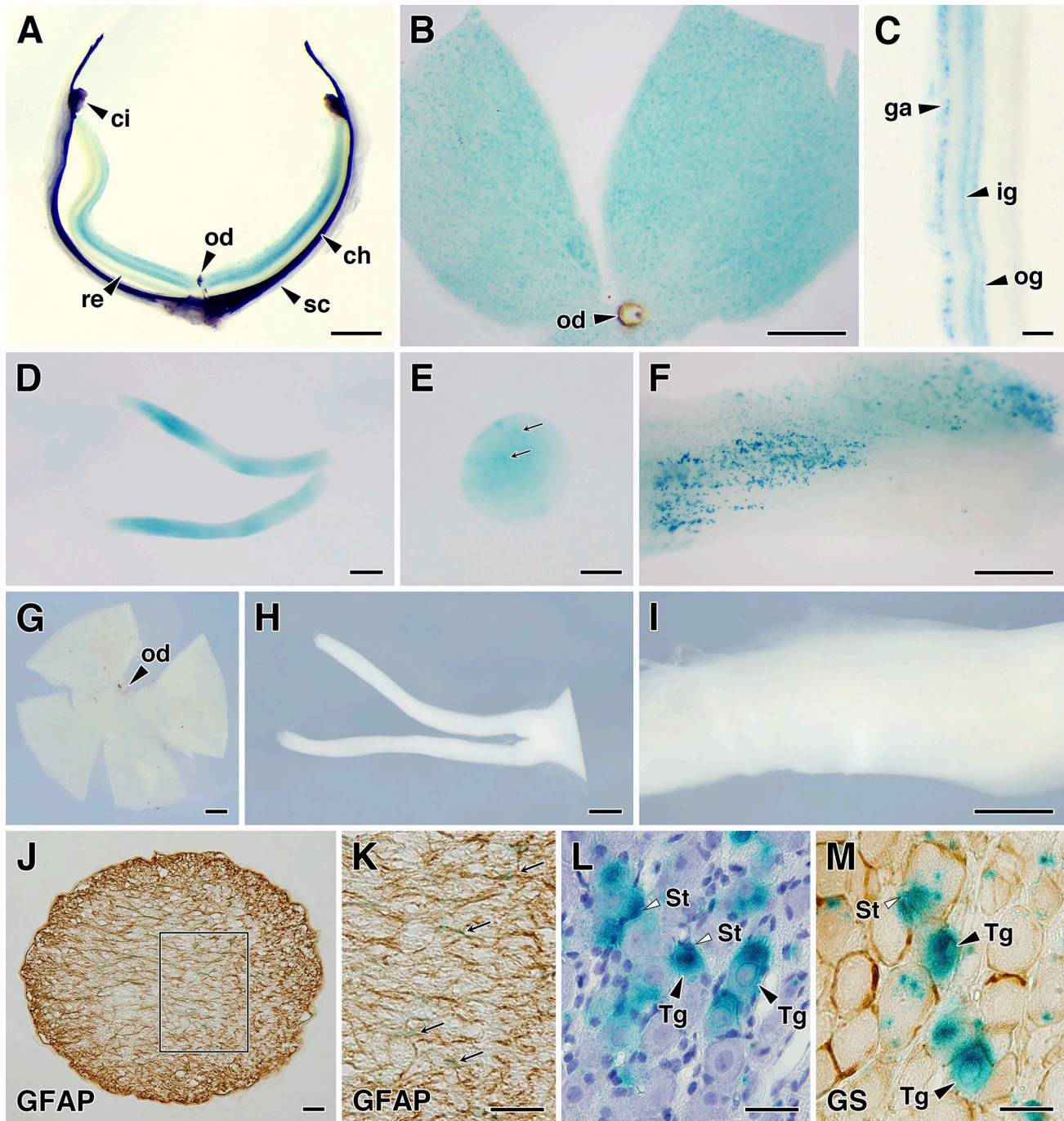


Figure 7. *LacZ* expression in the peripheral nervous tissues of *Sgll1-gtKO* mice. In the eye, *LacZ* expression was observed in the retina. The panels show a horizontal slice of the eye (A), a flat-mounted retinal preparation (B), and a transverse retinal section (C). *LacZ* is expressed in the ganglion cell layer and inner and outer granular layers. In the peripheral nervous system, *LacZ* expression was observed in the optic nerves (D and E) and trigeminal ganglion (F). Panels (D) and (E) show the shape and a transverse section of the optic nerve. Panels (G)–(I) show the negative control of *LacZ* staining in WT mice tissues: a flat-mounted retina (G), optic nerves (H), and trigeminal ganglion (I). Panels (J) and (K) show GFAP immunolabeling of the optic nerve transverse section and (K) shows an enlarged image of the rectangle enclosed by the line in (J). The arrows indicate *LacZ*-positive signals in glial cells in (E) and GFAP-immunolabeled astrocytes in (K). The trigeminal ganglion sections were counterstained with hematoxylin (L) or reacted with an anti-GS antibody (M). Scales, 500 μm (A, B, D, F, and G–I), 100 μm (C and E), and 20 μm (J to M). Abbreviations: ch, choroid; ci, ciliary body; ga, ganglionic cell layer; ig, inner granular layer; od, optic disk; og, outer granular layer; re, retina; sc, sclera; St, satellite cells; Tg, trigeminal ganglion neuron; *Sgll1*, secretogranin III; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase.

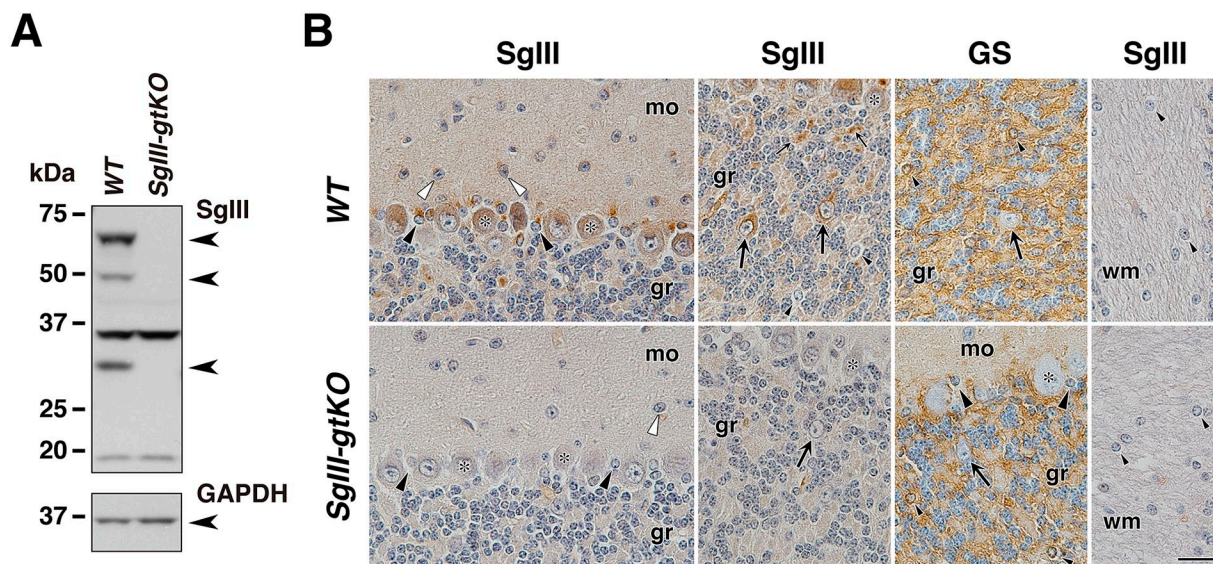


Figure 8. Expression of SgIII in the cerebellum. (A) Cerebellar tissue lysates (40 μ g of protein extract) from WT and *SgIII-gtKO* mice were used for SDS-PAGE, followed by immunoblotting using anti-SgIII and control anti-GAPDH antibodies. Three SgIII-specific bands were detected (arrowheads), and two bands near 37 and 20 kDa were nonspecific. Immunohistochemistry using anti-SgIII (B, C, and E) and anti-GS (D) antibodies. The asterisks indicate Purkinje cells, and the large black arrowheads indicate Bergmann glia in the piriform neuron layer. The open arrowheads indicate basket-like cells. The large arrows indicate Golgi-like cells that do not express GS, and the small arrows indicate the neuropil surrounding the granule cell perikaryon (considered to be the cerebellar glomerulus). The small black arrowheads indicate GS-positive astrocytes in which SgIII expression was not detected in the granular layer. Scale, 20 μ m. Abbreviations: gr, granular layer; mo, molecular layer; wm, white matter; *SgIII*, secretogranin III; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; WT, wild-type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the roles of astrocytes, synaptic glutamate clearance is one of the critical functions in regulating neurotransmitter homeostasis.²⁸ Therefore, we treated two rat glioma-derived cell lines with similar properties, C6 and RGC-6, with glutamate to investigate the relationship between the changes in SgIII expression level and glutamate stimulation. Although the basal level of SgIII expression was very low in these glioma cells compared with that detected in the control pituitary tissue (Fig. 9A), SgIII expression was significantly induced (1.5–2.0-fold) by glutamate treatment (Fig. 9B).

Discussion

We recently established the *SgIII-gtKO* mouse line and demonstrated that these mice exhibit maladaptation to an inadequate diet and stress because of the impairment of the proteolytic conversion of prohormones in SGs, although the biogenesis of SGs and basal secretion of hormones are not impaired in ordinary conditions. After high-calorie-diet feeding, *SgIII-gtKO* mice showed marked obesity, which was associated with increased proinsulin accumulation in pancreatic islets and decreased active insulin secretion. Moreover, after loading with chronic restraint stress, the plasma ACTH concentration was not sufficiently increased, and the

accumulation of proopiomelanocortin (POMC), which is an ACTH precursor in the pituitary gland, was increased. Based on these results, it was suggested that SgIII is of great importance in the process of conversion to active hormones by limited prohormone degradation during SG formation, especially in the peptide-hormone-secreting endocrine cells.¹⁸

We report here the findings obtained from *SgIII-gtKO* mice, in which *LacZ* gene expression is controlled by the authentic *SgIII* promoter. We analyzed the *LacZ* expression macroscopically and identified cells/tissues that had not been analyzed to date at the microscopic level. The *LacZ* staining of the tissues indicated the cell types that endogenously express the SgIII protein. In the canonical endocrine tissues (pituitary glands, adrenal glands, and pancreatic islets), the expression of the SgIII protein and that of *LacZ* were well matched in immunoblotting and histochemical analyses, suggesting the advantages of the gene trap method. Although the details of SgIII expression verified by in situ hybridization and immunohistochemistry in the rat CNS have been significantly reported,¹¹ the presentation of topographical images is limited. Therefore, we planned to analyze *LacZ* expression as a reporter in the mouse CNS and clarify its relative expression intensity. The comprehensive analysis

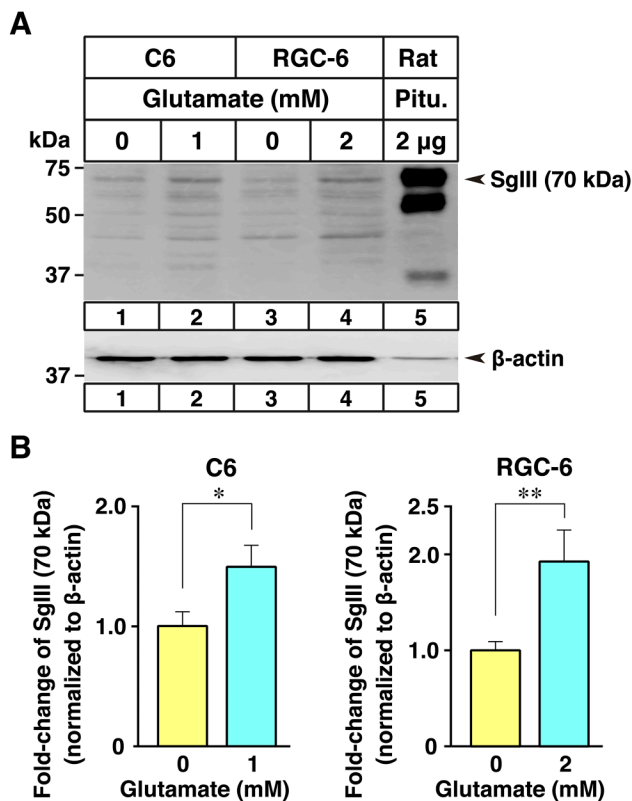


Figure 9. Expression of SgIII in C6 and RGC-6 glioma cells cultured with glutamate. (A) Immunoblot analysis of SgIII (70 kDa) and β -actin in the total cell lysates (20 μ g) from C6 and RGC-6 cells cultured with the indicated concentration of glutamate for 24 hr and in rat pituitary lysates (2 μ g). (B) Quantification of the immunoblot of SgIII in C6 and RGC-6 cells. The intensities of the immunoreactive bands on the immunoblots presented in (A) were quantified by densitometric analysis. The expression levels of each protein were normalized to that of β -actin and are expressed as arbitrary units, whereas the mean levels in 0 mM glutamate are defined as 1.0. Data are presented as mean \pm SEM for each group ($n=6$ per group; six sample preparations from six independent experiments). * $p<0.05$; ** $p<0.01$. Abbreviation: SgIII, secretogranin III.

performed in the nervous tissues showed an extensive expression of *LacZ* in various brain regions, including the cerebral cortex, caudate putamen, hypothalamus, hippocampus, thalamus, amygdala, midbrain, cerebellum, pons, medulla oblongata, and gray matter of the spinal cord. The strongest expression level was observed in the cerebral cortex, hippocampus, and cerebellar cortex. The typical SgIII expression in neurons with neuroendocrine properties, such as those located in the hypothalamic nuclei, as well as in the cerebral cortical pyramidal neurons, hippocampal pyramidal neurons, and cerebellar Purkinje neurons has been reported.^{11,19} We recently detected SgIII expression in active amine-secreting cells in the

gastrointestinal tract and adrenal medulla.¹⁷ The somas of amine-secreting neurons are present in the hypothalamic arcuate nuclei, substantia nigra, central gray matter (catecholamine neurons), and area postrema (serotonin neurons) in the CNS, and a coherent *LacZ* expression in these brain regions was observed. Furthermore, we found that SgIII is expressed in the ependymal cells that constitute the ventricular wall as a new finding. We then immunohistochemically analyzed SgIII expression in the cerebellum in detail because these cellular elements have not been fully elucidated. We found that SgIII was expressed in Golgi-like cells and basket-like cells, in addition to the previously reported Purkinje cells and Bergmann glia. This was the first report that at least four different cell types express SgIII in the cerebellum. SgIII expression was also observed in presumable cerebellar glomeruli in the granular layer. In the peripheral nervous system, *LacZ* expression was observed in retinal neurons and in astrocytes in the optic nerves. Furthermore, we found that *LacZ* was expressed in subtypes of ganglion neurons and satellite cells (ganglionic glial cells) in the trigeminal ganglion.

Regarding the cellular elements that express SgIII in the CNS, an important study reported that SgIII is a glial cell granin, the overexpression of which is accompanied by reactive gliosis.¹⁶ SgIII expression in astrocytes, but not in oligodendrocytes, has been reported in the rat somatosensory cortex, hippocampal CA1, corpus callosum, optic nerve, cerebellar granular layer, and Bergmann glia. The expression levels of SgIII in astrocytes were rather remarkable compared with those detected in neurons. Interestingly, transcriptional regulation of the astrocytic expression of SgIII has been suggested to be associated with the trafficking and release of SgIII as secretions, and its upregulation was detected during reactive gliosis.¹⁶ We also observed *LacZ* expression in astrocytes distributed in the cerebral cortex, hippocampal DG and CA fields, medulla oblongata, and so on. *LacZ* was expressed abundantly in astrocytes. However, in the cerebellum, Bergmann glia showed a constitutive SgIII/*LacZ* expression, in contrast to their negligible expression in astrocytes localized in the granular layer and white matter. Thus, based on the results of this study, it also became clear that the expression level of SgIII varies among astrocyte subtypes in the physiological condition.

SgIII expression is not only detected in neurons but also in astrocytes, highlighting the essential diversity of this molecule. Even in neurons, there is a diversity in the localization of intracellular proteins.¹¹ For example, as shown by ultrastructural immunocytochemistry, the SgIII protein was localized in the perikaryon and fibers

in pyramidal cells of the cerebral cortex and seemed to be delivered at the site of presynaptic terminals, whereas SgIII was localized within the dendritic processes in the hippocampal CA3 pyramidal neurons. The intracellular localization of SgIII appeared to vary, from synaptic vesicles in the former to small organelles resembling vesicles of smooth endoplasmic reticulum in the latter. It is interesting to note that there are wide range differences from intracellular localization between neuron subtypes to varied expression levels among astrocyte subtypes.¹¹

Recently, several lines of research have suggested that SgIII is involved in the pathophysiology of nervous tissues. In patients with Alzheimer's disease (AD) and an AD transgenic mouse model, a remarkable accumulation of SgIII and CPE was detected in the cortical lesion site, where dystrophic neurites surrounding amyloid plaques and plaque-associated reactive astrocytes exist.²⁹ Because CPE and SgIII are essential in the processing and targeting of neuropeptides and neurotrophins, their participation in the pathological progression of AD may be suggested.³⁰ SgIII was expressed in the retina, where it acted as an angiogenic factor in diabetic retinopathy.^{31,32} Furthermore, SgIII has been suggested to be involved in signal transduction in neurotoxin-induced apoptosis in dopaminergic neuroblasts.³³ Although the pathophysiological significance of the astrocytic SgIII expression is not fully understood, this protein might be involved in the secretory function of astrocytes (recently also called gliocrine). It has been noted that extremely diverse vesicles and secretory organelles, such as synaptic-like microvesicles, dense-core vesicles, lysosomes, exosomes, and ectosomes, are secreted from astrocytes.^{34,35} Furthermore, astrocytes are traditionally involved in the clearance of the neurotransmitter glutamate as a physiological function.²⁸ In this regard, we analyzed the changes in SgIII expression level after stimulation of glioma-derived cell lines with glutamate and demonstrated that exposure to glutamate increased SgIII expression. SgIII upregulation is also suggested to be involved in the parkinsonian toxin-induced astrocyte activation, and SgIII may act as a cofactor in astrocyte activation stimulated by various toxins.^{36,37} Thus, it seems that the action of SgIII as an active substance also has potential importance. Moreover, the fragmentation pattern of SgIII from its precursor in the nervous tissues showed a similar proteolytic processing to that detected in the adrenal gland, which was different from that of other peptide-hormone-secreting endocrine cells.

In summary, we performed an extensive analysis of *LacZ* gene expression in *SgIII-gtKO* gene-trapped

mice. We demonstrated that this descriptive mapping of the SgIII-expressing cells is significant to provide a more precise localization of this protein in the central and peripheral nervous systems. The comprehensive pursuit of this reporter gene expression allowed us to clarify the previously unpredictable cell types that express SgIII with sufficient sensitivity and confirmed what is already known about the distribution of SgIII cellular expression by immunohistochemistry and in situ hybridization techniques.

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Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

HG designed the study, carried out the histological and biochemical experiments, performed the data analysis, and drafted the manuscript. MH and AH prepared mice samples, and performed biochemical analysis and glioma culture experiments. TY and ST contributed to the experimental design, data analysis, and reviewing of the manuscript. All authors have read and approved the final manuscript.

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