

RESEARCH ARTICLE

Voltage-gated calcium channel $\alpha_2\delta$ -1 subunit is involved in the regulation of glucose-stimulated GLP-1 secretion in mice

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Abstract

Glucagon-like peptide-1 (GLP-1) is an incretin produced by enteroendocrine preproglucagon (PPG)-expressing cells in response to nutrient ingestion that potentiates insulin secretion. The voltage-gated Ca^{2+} channel has been reported previously to be involved in glucose-stimulated GLP-1 secretion; in this study, we show that PPG-cells in upper and lower small intestine substantially express the voltage-gated Ca^{2+} channel $\alpha_2\delta$ -1 subunit ($\text{Ca}_v\alpha_2\delta$ -1). In vitro experiments using NCI-H716 cells demonstrate that inhibition of $\text{Ca}_v\alpha_2\delta$ -1 by gabapentin (GBP), an inhibitory ligand of the $\alpha_2\delta$ subunit, attenuates glucose-stimulated intracellular calcium elevation and reduces GLP-1 secretion. In addition, systemic administration of gabapentin significantly reduces glucose-stimulated GLP-1 secretion without affecting blood glucose levels in wild-type mice. Furthermore, knockout mice of intestine-specific *Cacna2d1*, a gene encoding $\text{Ca}_v\alpha_2\delta$ -1, exhibit reduced GLP-1 secretion in response to oral glucose administration regardless of sex. These results demonstrate that $\text{Ca}_v\alpha_2\delta$ -1 expressed in PPG-cells plays an important role in glucose-stimulated GLP-1 secretion and represents a potential target in the treatment of diabetes and obesity.

NEW & NOTEWORTHY In this study, we establish high expression of the voltage-gated Ca^{2+} channel $\alpha_2\delta$ -1 subunit ($\text{Ca}_v\alpha_2\delta$ -1) subunit in enteroendocrine glucagon-like peptide-1 (GLP-1) producing cells and elucidate its role in GLP-1 secretion, providing a more detailed understanding of the mechanism of GLP-1 secretion.

glucagon-like peptide-1 (GLP-1); incretin; voltage-gated Ca^{2+} channel $\alpha_2\delta$ -1 subunit ($\text{Ca}_v\alpha_2\delta$ -1)

INTRODUCTION

Glucagon-like peptide-1 (GLP-1) is an incretin, the secretion of which has been shown to be related to nutrient intake (1). In addition to its glucose-dependent insulinotropic effect, GLP-1 has a wide variety of physiological functions that promote normoglycemia, including suppression of glucagon secretion (2), inhibition of gastric emptying (3), and facilitation of satiety (4, 5). Based on the physiological properties of GLP-1, dipeptidyl peptidase-4 (DPP-4) inhibitors and DPP-4-resistant GLP-1 mimetics are being used clinically to treat type 2 diabetes by inhibiting the rapid natural degradation of the hormone. In addition, some GLP-1 receptor agonists are being used to treat obesity in patients without diabetes (6). Thus, further elucidation of the GLP-1 secretory mechanism may well lead to the development of new treatments for both type 2 diabetes and obesity.

GLP-1 is secreted from enteroendocrine preproglucagon-expressing (PPG) cells in response to various stimuli including luminal glucose (7). Previous in vitro studies have shown that

glucose transport is mediated by the apical sodium-glucose cotransporter 1 (SGLT1) in the GLP-1-producing cells through electrogenic coupling of Na^+ influx, which results in membrane depolarization and voltage-gated Ca^{2+} entry (7, 8). The mechanism by which enteroendocrine PPG-cells sense glucose through SGLT1 and trigger influx of Ca^{2+} to secrete GLP-1 has been further described in vivo (9, 10); a recent study on human tissue has confirmed this finding (11).

Voltage-gated Ca^{2+} (Ca_v) channels are heteromeric complexes comprising $\text{Ca}_v\alpha_1$, the main subunit forming the ion-conducting pore, and three differing auxiliary subunits, i.e., $\text{Ca}_v\alpha_2\delta$, $\text{Ca}_v\beta$, and $\text{Ca}_v\gamma$ (12, 13). The $\text{Ca}_v\alpha_2\delta$ subunit plays a very important role in controlling trafficking of the α_1 subunit and cell-surface expression of the channels, which produces increased Ca^{2+} current density (12, 14–16). Four $\text{Ca}_v\alpha_2\delta$ subunit genes, i.e., *Cacna2d1*–*Cacna2d4*, have been identified and found to be expressed in several cell types including endocrine cells (17, 18). Previous studies showed that $\text{Ca}_v\alpha_2\delta$ -1 (encoded by *Cacna2d1* gene; gene names are italicized and protein names are not, the same hereinafter) is the main



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$\text{Ca}_v\alpha_2\delta$ isoform expressed in both human and mouse pancreatic islets (19); systemic *Cacna2d1* deletion in mice leads to glucose intolerance due to reduced-insulin release and increased susceptibility to diabetes (20). Another recent report confirms that $\text{Ca}_v\alpha_2\delta\text{-1}$ affects glucose-stimulated insulin secretion by regulating transport of the Ca_v channels to the plasma membrane (21). However, there are no reports on the contribution of the $\text{Ca}_v\alpha_2\delta$ subunit to the secretory mechanism of enteroendocrine hormones including GLP-1.

In the present study, we demonstrate the high expression level of $\text{Ca}_v\alpha_2\delta\text{-1}$ in PPG-cells and analyze changes in glucose-stimulated GLP-1 secretion by inhibiting $\text{Ca}_v\alpha_2\delta\text{-1}$ activity by using gabapentin (GBP), an inhibitory ligand of the $\text{Ca}_v\alpha_2\delta$ subunit, and intestine-specific *Cacna2d1* knockout mice. Our findings show that $\text{Ca}_v\alpha_2\delta\text{-1}$ plays an important role in the regulation of glucose-stimulated GLP-1 secretion and suggest a novel target for drug development for the treatment of diabetes and obesity.

MATERIALS AND METHODS

Animals

The animals used in this study were male and female C57BL/6J mice maintained under conditions of a 12-h light and dark cycle and fed control-fat diet (CFD; 10% fat, 20% protein, and 70% carbohydrate by energy) (Research Diets Inc., New Brunswick, NJ) with free access to water and food unless otherwise indicated. The mice used in individual experiments were age-matched at the beginning of the experiments. Glucagon-green fluorescent protein (GFP) knock-in (*Gcg^{gfp} +/−*) mice in which the PPG-cells are labeled by enhanced green fluorescent protein (EGFP) were generated as reported previously (22) and back-crossed with the C57BL/6J strain for more than 10 generations. Glucose-dependent insulintropic polypeptide (GIP)-GFP heterozygous knock-in (*GIP^{gfp} +/−*) mice were generated as described previously (23). $\alpha_2\delta\text{-1}$ -floxed mutant mice (RRID:IMSR_JAX:030704) and Villin-Cre transgenic mice (RRID:IMSR_JAX:004586) were purchased from The Jackson Laboratory. Recently, we have reported that villin is expressed in chromogranin A, cholecystokinin (CCK), GLP-1, GIP, and peptide YY (PYY)-expressing cells and have successfully generated intestine-specific G protein-coupled receptor (GPR) 120 knockout mice by breeding GPR120-floxed mice and Villin-Cre transgenic mice (24). In this study, intestine-specific *Cacna2d1* knockout (CKO) mice were generated by breeding homozygous-floxed *Cacna2d1* mice with Villin-Cre transgenic mice. All experiments used age-matched CKO (*Cacna2d1^{LoxP/LoxP}; Cre +/−*) and $\alpha_2\delta\text{-1}$ -floxed mice as littermate controls. All animal protocols were approved by the Animal Care Committee of Kyoto University (Med Kyo 16584).

Isolation of PPG-Cells from Intestinal Epithelium of *Gcg^{gfp} +/−* Mice

The whole length of the small intestine was taken from the anal side of the pyloric ring to the oral side of the cecum, with the oral half of the whole length as the upper small intestine and the anal half as the lower small intestine, from which specimens were prepared. The procedure of PPG-cell isolation from murine intestinal epithelium was described

previously (25). Briefly, the small intestine of 10-wk-old *Gcg^{gfp} +/−* mice was removed, cut into several round pieces, and tied on one side with a thread. This pouch-like portion of intestine was injected with Hanks' balanced salt solution (HBSS) containing 0.5 mg/mL collagenase, clamped, and incubated with CO_2 at 37°C for 10 min in Krebs-Ringer bicarbonate buffer (KRBB: 120 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 2.4 mM CaCl_2 , 20 mM NaHCO_3). The digested intestinal epithelium was cultured in a humidified incubator (95% air and 5% CO_2) at 37°C for 1 h. Afterward, it was centrifuged at 180 g for 5 min, resuspended in phosphate-buffered saline (PBS) twice, and filtered with a cell strainer (352340, Falcon cell strainer, BD Biosciences, San Jose, CA). The size of the openings of the strainer was 40 μm . GFP-positive cells in the intestinal epithelium were isolated using the BD FACS Aria™ flow cytometer (BD Biosciences, San Jose, CA). Sorted cells were collected into vials containing medium. Total RNA was extracted from sorted cells of *Gcg^{gfp} +/−* mouse intestinal epithelium using the PicoPure RNA isolation kit (Applied Biosystems, Inc., Alameda, CA) and treated with DNase (Qiagen Inc., Valencia, CA). GIP-producing cells were also isolated from small intestine of *GIP^{gfp} +/−* mice using the same technique as described earlier.

Quantitative Reverse Transcription Polymerase Chain Reaction

Complementary DNA (cDNA) was prepared by reverse transcriptase (Invitrogen, CA) with an oligo (dT) primer (Invitrogen, CA). Messenger RNA (mRNA) levels were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using an ABI PRISM 7000 Sequence Detection System (AB StepOne Plus Real-Time PCR; Applied Biosystems, Foster City, CA). PCR analyses were carried out using oligonucleotide primers. SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) was prepared for PCR runs. Thermal cycling conditions were denaturation at 95°C for 10 min followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. To quantify the results obtained by qPCR, we used the standard curve method with ribosomal protein S18 (*Rps18*) as an internal control. Primer pairs for PCR were as follows: *Gcg* forward, 5'-TGAAGACAAACGCCACTCAC-3', and reverse, 5'-TCATGACGTTTGGCAATGTT-3'; *Cacna2d1* forward, 5'-CTT-CGCCGCTCACTATCAAGT-3', and reverse, 5'-CCAGTTGG-CGTGCATTGTTG-3'; *Rps18* forward, 5'-AAGTTCCAGCAC-ATTTTGCG-3', and reverse, 5'-TGATGGTGATCACTCG-CTCC-3'.

Immunofluorescence

The tissue samples of 10-wk-old wild-type mice, *Gcg^{gfp} +/−* mice, *GIP^{gfp} +/−* mice, $\alpha_2\delta\text{-1}$ -floxed (Floxed) mice, and CKO mice were fixed in 10% formalin solution and embedded in paraffin. The whole length of the small intestine was taken from the anal side of the pyloric ring to the oral side of the cecum, with the oral half of the whole length as the upper small intestine and the anal half as the lower small intestine, from which specimens were prepared. To examine localization of $\text{Ca}_v\alpha_2\delta\text{-1}$ in the small intestine, rehydrated paraffin sections of *Gcg^{gfp} +/−* mice and *GIP^{gfp} +/−* mice were incubated overnight at 4°C with primary rabbit anti-GFP antibody

(Santa Cruz Biotechnology, sc-9996, 1:200) and mouse anti-CACNA2D1 antibody (Thermo Fisher Scientific, MA3-921, 1:200). To confirm the specific loss of expression of $\text{Ca}_v\alpha_2\delta\text{-1}$ in intestinal GLP-1-producing cells in CKO mice, rehydrated paraffin sections of Floxed mice and CKO mice were incubated overnight at 4°C with primary rabbit anti-GLP-1 antibody (Abcam, ab111125, 1:10,000) and mouse anti-CACNA2D1 antibody. After the overnight incubation with primary antibodies, the sections were incubated for 1 h at room temperature with secondary antibody. Details of secondary antibodies were as follows: Alexa Fluor 546 Goat Anti-Mouse (Life Technologies Japan Corp., Tokyo Japan, A-11030) for anti-CACNA2D1 antibody and Alexa Fluor 488 Goat Anti-Rabbit (Life Technologies Japan Corp., Tokyo, Japan, A11034) for anti-GFP antibody and anti-GLP-1 antibody. Images were taken using fluorescent microscopy with the FSX100 system (Olympus Corporation, Tokyo, Japan).

Prepared NCI-H716 cells (RRID:CVCL_1581) were placed on a 0.1% gelatin-coated glass coverslip for 10 min at room temperature before fixation with 4% formaldehyde in PBS solution. After fixation, the cells were blocked by 1-h incubation in 2% BSA solution and incubated overnight at 4°C with mouse anti-CACNA2D1 antibody (Thermo Fisher Scientific, MA3-921, 1:200). The coverslip with cells were incubated for 1 h at room temperature with secondary antibody on the second day.

Culture of NCI-H716 Cells and Secretion Studies

Human enteroendocrine NCI-H716 cells (RRID:CVCL_1581) were purchased from American type culture collection (Manassas, VA) and cultured in RPMI-1640 (Fujifilm Wako, 189-02025) supplemented with 10% heat-inactivated fetal calf serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere (5% CO₂ and 95% air). NCI-H716 cells were plated on 4% Matrigel-coated (Corning, 354234) 24-well dishes (2 × 10⁵/well) and incubated with different concentrations of gabapentin (GBP), an inhibitory ligand of the $\text{Ca}_v\alpha_2\delta\text{-1}$ subunit, for 40 h. The medium was then replaced with 500 µL of KRB buffer containing 3% glucose and the differing concentrations of GBP. All samples were collected after 2-h incubation and centrifuged (10,000 g) for 5 min. Hydrochloric acid (0.1 N) was added to each well and rotated on ice for 10 min to lyse the cells. Samples to measure GLP-1 content were collected and centrifuged (3,000 g) for 10 min. The supernatant was then collected to measure the total GLP-1 concentration using an enzyme-linked immunosorbent assay kit (Sigma-Aldrich, Merck Millipore, EZGLP1T-36K). Protein content of the cell homogenate was determined using the Bradford assay (Bio-Rad, Inc.).

Measurement of Intracellular Ca²⁺ Concentration

NCI-H716 cells were plated on 4% Matrigel-coated 96-well dishes (5 × 10⁴/well) and incubated with different concentrations of GBP for 40 h. The cells were then loaded with 5 µM fura-2AM (Dojindo, Japan) in KRB buffer for 30 min at 37°C. After incubation, the NCI-H716 cells were washed twice by KRB buffer and placed on a microplate reader (SynergyH1; BioTek instruments). Images through the filter sets (340/11,

380/20 -DM 400- 508/20) were obtained every 30 s at 37°C and the 340 nm/380 nm fluorescence ratio in each well was recorded.

Oral Glucose Administration

To evaluate the effect of pharmacological inhibition of $\text{Ca}_v\alpha_2\delta\text{-1}$ on GLP-1 secretion in vivo, wild-type mice were treated with intraperitoneal injections of either GBP (300 mg/kg) or vehicle (PBS) after a 16-h fasting period. After another 2 h, an oral glucose tolerance test (OGTT) was performed. Blood samples were collected at 0, 15, and 30 min after oral glucose (6 g/kg body wt) administration. To confirm the contribution of $\text{Ca}_v\alpha_2\delta\text{-1}$ expressed in enteroendocrine cells to glycemic regulation, blood samples were collected from control mice and CKO mice at 0, 15, 30, 60, 90, and 120 min after oral glucose (6 g/kg body wt) administration. Blood glucose levels at 0, 15, 30, 60, 90, and 120 min after glucose load were measured by the glucose oxidase method. Insulin, total GLP-1, and total GIP levels at 0, 15, 30, and 60 min after glucose load were measured by using enzyme-linked immunosorbent assay kits as follows: insulin kit (Wako Fujifilm, Japan, 296-89801), total GLP-1 kit (Sigma-Aldrich, Merck Millipore, EZGLP1T-36K), and total GIP kit (Sigma-Aldrich, Merck Millipore, EZRMGIP-55K), respectively.

Statistical Analysis

The data are presented as means ± standard deviation (SD). Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by a post hoc analysis for OGTT experiments and Student's *t* test for others. *P* values <0.05 were considered statistically significant. Data analyses and visualizations were conducted using GraphPad Prism software (v.8.0.1, GraphPad, La Jolla, CA).

RESULTS

$\text{Ca}_v\alpha_2\delta\text{-1}$ Is Highly Expressed in GLP-1-Producing Cells

We performed RT-PCR of PPG-cells and immunohistochemistry of the small intestine from *Gcg^{flp/+}* mice. GFP-positive cells isolated from the small intestine of *Gcg^{flp/+}* mice showed significantly higher proglucagon gene expression than GFP-negative cells, confirming that GLP-1-producing cells (PPG-cells) were isolated as intended (Fig. 1A). *Cacna2d1* mRNA expression levels in PPG-cells were significantly higher compared with those in non-PPG-cells both in upper and lower small intestine (Fig. 1B). The mRNA expression levels of the other $\text{Ca}_v\alpha_2\delta$ subunit genes, *Cacna2d2*, *Cacna2d3*, and *Cacna2d4*, were extremely low in PPG-cells and did not differ significantly from those in non-PPG-cells (data not shown), indicating that $\text{Ca}_v\alpha_2\delta\text{-1}$ is the main $\text{Ca}_v\alpha_2\delta$ isoform expressed in PPG-cells. Immunohistochemical staining of the upper and lower small intestine of *Gcg^{flp/+}* mice showed that $\text{Ca}_v\alpha_2\delta\text{-1}$ and EGFP proteins were expressed in the same cell (Fig. 1C). We performed double-staining of mouse small intestine with antibodies for $\text{Ca}_v\alpha_2\delta\text{-1}$ and GLP-1, demonstrating that GLP-1-expressing cells in upper and lower small intestine indeed express $\text{Ca}_v\alpha_2\delta\text{-1}$ (Fig. 1D). These data indicate the high expression levels of *Cacna2d1* mRNA and $\text{Ca}_v\alpha_2\delta\text{-1}$ protein in GLP-1-expressing cells. On the other hand, CACNA2D1-positive/GLP-1-negative cells

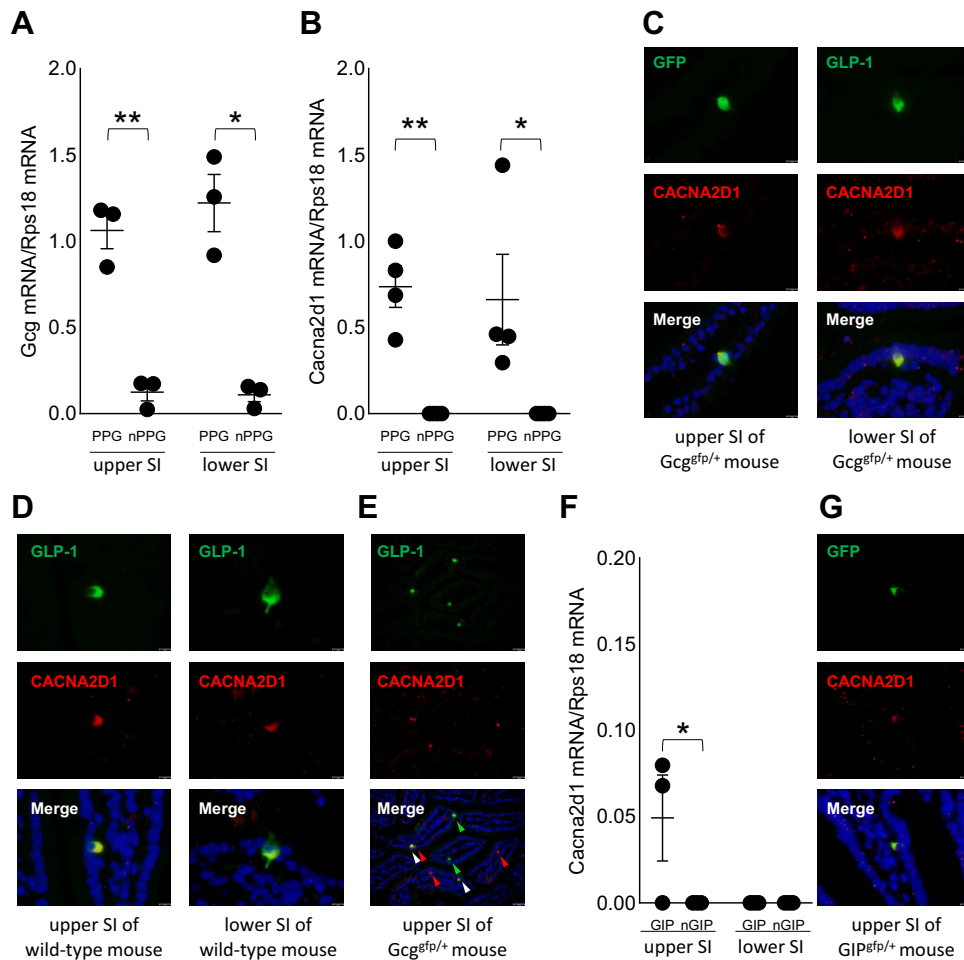


Figure 1. Expression of voltage-gated Ca²⁺ channel α₂δ-1 subunit (Ca_vα₂δ-1) subunit in enteroendocrine preproglucagon (PPG)-expressing cells. **A:** expression levels of *proglucagon* (*Gcg*) mRNA in PPG-cells (PPG) and non-PPG-cells (nPPG) isolated from upper small intestine (SI) and lower SI. Data are expressed as the ratio to that of ribosomal protein S18 (Rps18) in the same sample. **P* < 0.05, ***P* < 0.01 between non-PPG-cells and PPG-cells. *n* = 4 for each group. Data are expressed as means ± SD. Student's *t* test was used. **B:** expression levels of *Cacna2d1* mRNA in PPG-cells (PPG) and non-PPG-cells (nPPG) isolated from upper and lower SI. Data are expressed as the ratio to that of Rps18 in the same sample. **P* < 0.05, ***P* < 0.01 between non-PPG-cells and PPG-cells. *n* = 4 for each group. Data are expressed as means ± SD. Student's *t* test was used. **C:** immunohistochemical staining for green fluorescent protein (GFP) (green) and Ca_vα₂δ-1 subunit (CACNA2D1) (red) in the upper and lower SI of a *Gcg*^{Gfp/+} mouse (*n* = 6, including 2 biological replicates and 3 technical replicates). **D:** immunohistochemical staining for glucagon-like peptide-1 (GLP-1) (green) and CACNA2D1 (red) in the upper and lower SI of a wild-type mouse (*n* = 6, including 2 biological replicates and 3 technical replicates). **E:** immunohistochemical staining for GFP (green) and CACNA2D1 (red) in the upper SI of a *Gcg*^{Gfp/+} mouse (low magnification view) (*n* = 6, including 2 biological replicates and 3 technical replicates). GFP-positive/CACNA2D1-positive cell (white arrow), GFP-positive/CACNA2D1-negative cell (green arrow), GFP-negative/CACNA2D1-positive cell (red arrow). **F:** expression levels of *Cacna2d1* mRNA in glucose-dependent insulintropic polypeptide (GIP)-producing cells (GIP) and non-GIP cells (nGIP) isolated from upper and lower SI of *GIP*^{Gfp/+} mice. Data are expressed as the ratio to that of Rps18 in the same sample. **P* < 0.05 between GIP-producing cells and non-GIP-producing cells. *n* = 4 for each group. Data are expressed as means ± SD. Student's *t* test was used. **G:** immunohistochemical staining for GFP (green) and CACNA2D1 (red) in the upper SI of a *GIP*^{Gfp/+} mouse (*n* = 6, including 2 biological replicates and 3 technical replicates).

and CACNA2D1-negative/GLP-1-positive cells were also observed, although in very small numbers (Fig. 1E), indicating that the expression of *Cacna2d1* is not exclusively confined to GLP-1-producing cells. *Cacna2d1* mRNA expression levels in GIP-producing-cells were significantly higher compared with those in non-GIP-producing-cells in upper small intestine, but not in lower small intestine (Fig. 1F). Immunohistochemical staining of the small intestine of *GIP*^{Gfp/+} mice showed that Ca_vα₂δ-1 and EGFP proteins were expressed in the same cell in upper small intestine (Fig. 1G), demonstrating that GIP-producing cells express Ca_vα₂δ-1. The lower small intestine inherently contains fewer GFP-positive cells than the upper small intestine,

and no cells positive for both GFP and CACNA2D1 were identified (data not shown).

Ca_vα₂δ-1 Inhibition Decreases Glucose-Stimulated Intracellular Ca²⁺ Elevation and GLP-1 Secretion

Immunofluorescence staining demonstrated the expression of Ca_vα₂δ-1 in human enteroendocrine NCI-H716 cells, a qualified cell line frequently used in GLP-1 secretion experiments (Fig. 2A). We first performed a 2-h preincubation with GBP to test the short-term effects, but found no change in GLP-1 secretion from NCI-H716 cells compared with the vehicle alone (data not shown). To ascertain the chronic effect

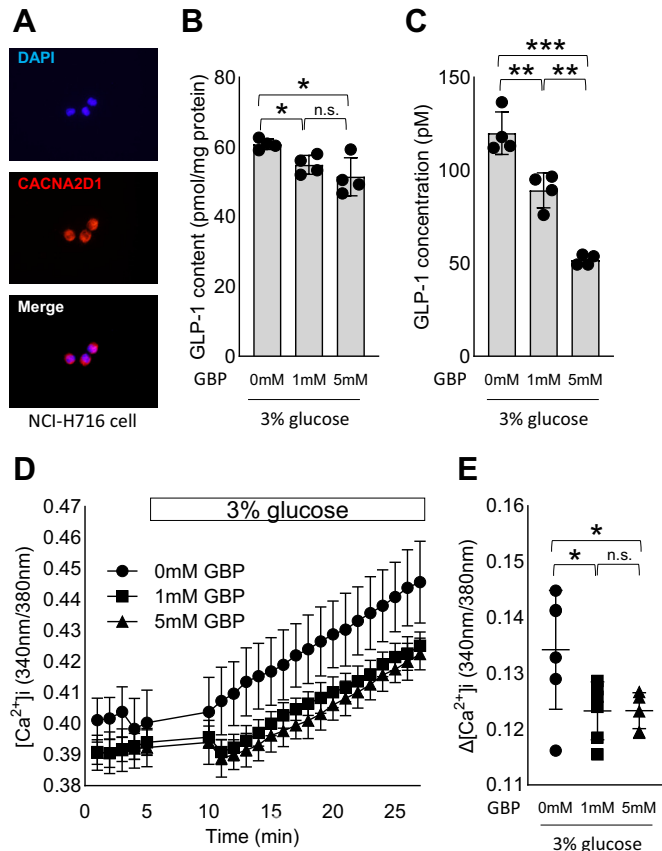


Figure 2. The effect of voltage-gated Ca²⁺ channel α₂δ-1 subunit (Ca_vα₂δ-1) subunit inhibition on intracellular Ca²⁺ elevation and glucagon-like peptide-1 (GLP-1) secretion in response to glucose. **A:** immunostaining for CACNA2D1 (red) and DAPI (blue) in NCI-H716 cells. **B:** GLP-1 content of NCI-H716 cells incubated with gabapentin (GBP). **P* < 0.05. *n* = 4 for each group. Data are expressed as means ± SD. **C:** GLP-1 secretion from NCI-H716 cells incubated with gabapentin (GBP). ***P* < 0.01, ****P* < 0.001. *n* = 4 for each group. Data are expressed as means ± SD. **D:** changes in intracellular Ca²⁺ concentrations ([Ca²⁺]_i) in NCI-H716 cells incubated with and without gabapentin (GBP) (0 mM GBP: black circle; 1 mM GBP: black square; 5 mM GBP: black triangle) in response to 3% glucose [0–5 min, Krebs-Ringer Buffer (KRB); 10–28 min, KRB + 3% glucose + gabapentin]; *n* = 6 for each group. Data are expressed as means ± SD. **E:** increment of fura-2 ratio (Δ[Ca²⁺]_i) in response to 3% glucose in conditions as in **A** [(Max. value during 5–28 min) – (Average value during 0–5 min)]. **P* < 0.05. *n* = 6 for each group. Data are expressed as means ± SD.

of GBP, we conducted a 40-h preincubation with GBP with reference to a previous report demonstrating that GBP inhibits Ca²⁺ currents only when applied chronically (26), and confirmed that GLP-1 secretion (Fig. 2C) and GLP-1 content (Fig. 2B) was significantly reduced by GBP at 3% glucose. There was no significant difference in the cell viability of NCI-H716 cells after a 2-h incubation in 3% glucose compared with that in 0% glucose (data not shown). GBP at concentrations of 1 mM and 5 mM significantly attenuated the increase in the glucose-stimulated intracellular calcium ions (Fig. 2, D and E) in NCI-H716 cells.

Inhibition of Ca_vα₂δ-1 Reduces Glucose-Stimulated GLP-1 Secretion in Wild-Type Mice

To evaluate the significance of Ca_vα₂δ-1 on glucose-stimulated GLP-1 secretion in vivo, we performed an OGTT

experiment 2 h after intraperitoneal injection of 300 mg/kg GBP or PBS in wild-type mice. The results showed that the blood GLP-1 concentration of mice in the GBP-treated group was significantly lower than that of the PBS-treated group 15 and 30 min after oral administration of glucose (Fig. 3B), whereas there was no significant difference in blood glucose levels between the two groups (Fig. 3A).

Glucose-Stimulated GLP-1 Secretion Is Decreased in Intestine-Specific *Cacna2d1* Knockout Mice

To clarify the significance of *Cacna2d1* deletion in enteroendocrine cells, we generated intestine-specific *Cacna2d1* knockout (CKO) mice. There was no significant difference in body weight between CKO mice and their littermates (data not shown). We performed immunohistochemistry of the small intestine and brain of control mice and CKO mice using anti-GLP-1 and anti-CACNA2D1 antibodies. Brain cells of CKO mice were positive for CACNA2D1 staining, as were those of control mice (Fig. 4A). On the other hand, GLP-1-positive cells in the small intestine of CKO mice were negative for CACNA2D1 staining whereas those of control mice were positive. Furthermore, no CACNA2D1-positive cells were identified in the small intestinal epithelium of CKO mice, confirming that CKO mice had intestine-specific loss of Ca_vα₂δ-1 expression (Fig. 4A). OGTT experimental results showed that the plasma GLP-1 concentration of CKO mice after oral administration of glucose for 15 min was significantly lower than that of controls, both in male and female mice (Fig. 4, D and H). GIP levels in male CKO mice were significantly lower compared with those in their littermate controls (Fig. 4E), whereas GIP levels in female CKO mice were not significantly different from those in control mice (Fig. 4I). Blood glucose levels and plasma insulin levels were similar in the control and mutant groups (Fig. 4, B, C, F, and G). These data indicated that genetic deletion of *Cacna2d1* in enteroendocrine cells resulted in reduced glucose-stimulated GLP-1 secretion regardless of sex.

DISCUSSION

In this study, we demonstrate that Ca_vα₂δ-1 is highly expressed in GLP-1-producing cells and is involved in GLP-

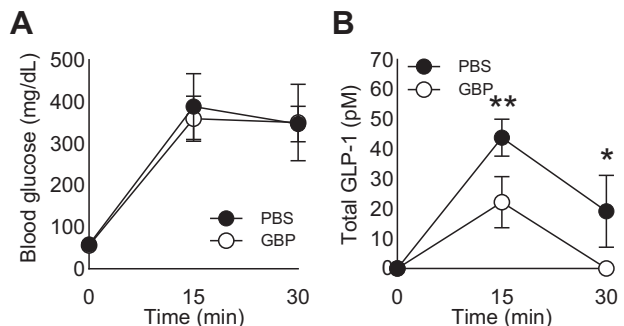


Figure 3. The effect of gabapentin (GBP) on glucose-stimulated glucagon-like peptide-1 (GLP-1) secretion in wild-type mice. Concentrations of blood glucose (A) and total GLP-1 (B) after oral administration of 6 g/kg glucose. PBS-treated mice (*n* = 4; filled circle) and GBP-treated mice (*n* = 3; unfilled circle). **P* < 0.05, ***P* < 0.01 between PBS-treated mice and GBP-treated mice. Data are expressed as means ± SD.

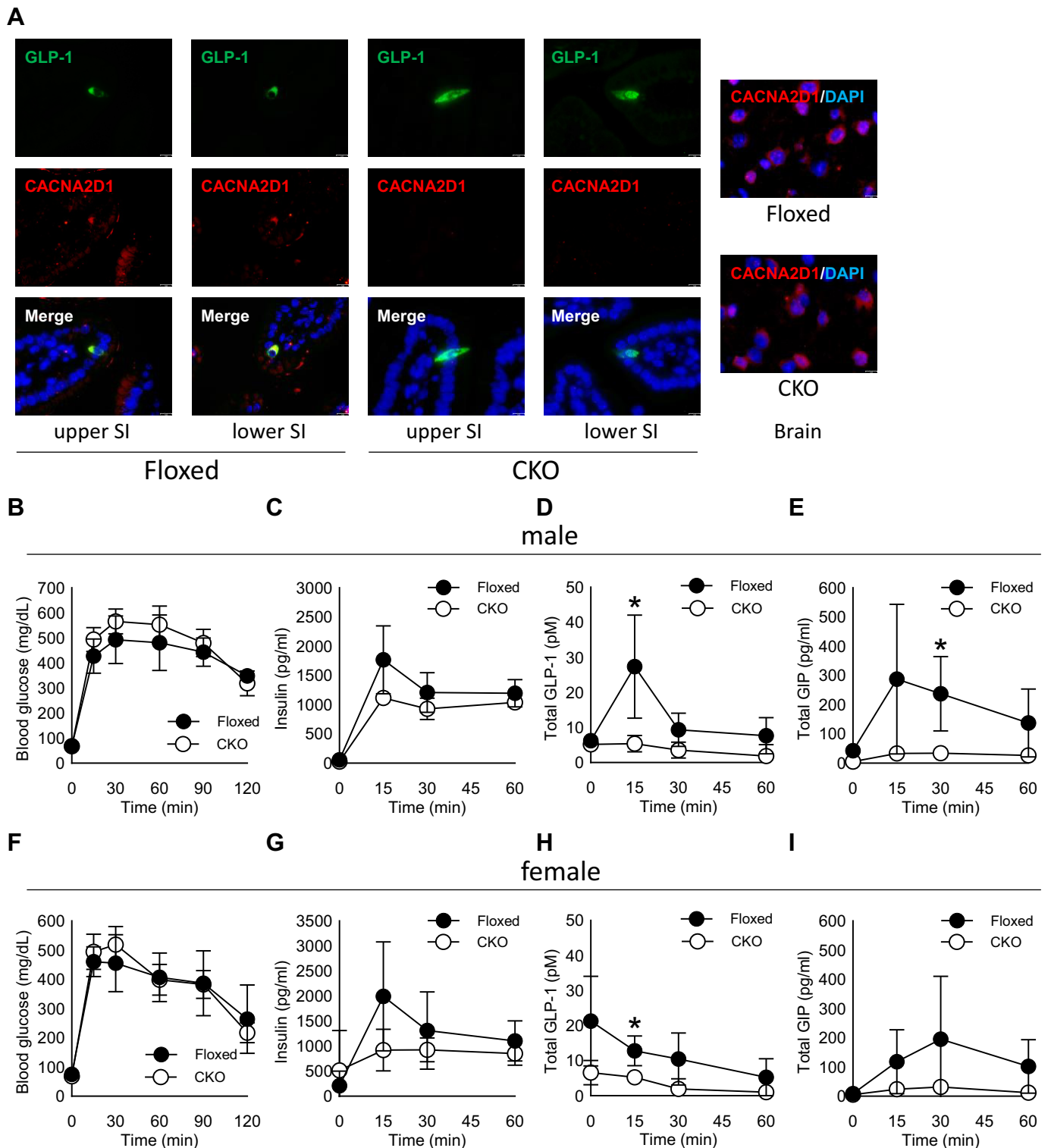


Figure 4. Glucagon-like peptide-1 (GLP-1) secretion in response to glucose in intestine-specific *Cacna2d1* knockout (CKO) mice. **A:** immunohistochemical staining for GLP-1 (green) and voltage-gated Ca²⁺ channel α₂δ-1 subunit (Ca_vα₂δ-1) (CACNA2D1) (red) in the upper and lower small intestine and brain of an α₂δ-1-floxed (Floxed) mouse and a CKO mouse (scale: 32 μm). Concentrations of blood glucose (**B** and **F**), insulin (**C** and **G**), total GLP-1 (**D** and **H**), and total glucose-dependent insulinotropic polypeptide (GIP) (**E** and **I**) after oral administration of 6 g/kg glucose in male mice (**B–E**) and female mice (**F–I**). **P* < 0.05. Floxed (*n* = 4–5; filled circle) and CKO (*n* = 3–4; unfilled circle). Floxed, α₂δ-1-floxed mice; CKO, intestine-specific *Cacna2d1* knockout mice. Data are expressed as means ± SD.

1 secretion in response to glucose via regulation of intracellular calcium concentrations.

The crucial role of Ca_v channels in GLP-1 secretion has been demonstrated previously by showing that nifedipine, an inhibitor of the L-type Ca^{2+} channel, attenuates glucose-stimulated GLP-1 secretion in GLUTag cells (27), in purified rat small intestine (10) and cultured human ileal tissue (11). It has also been reported that PPG-cells express the P/Q-type Ca^{2+} channel and that ω -agatoxin-IVA, a P/Q-type Ca^{2+} channel inhibitor, blocks Ca^{2+} currents in voltage clamp recordings of ileal organoid-derived PPG-cells (28). Interestingly, the only $\text{Ca}_v\alpha_2\delta$ subunit we could detect in isolated PPG-cells was $\text{Ca}_v\alpha_2\delta\text{-1}$ (data not shown), which is consistent with a previous report that the $\text{Ca}_v\alpha_2\delta\text{-1}$ subunit dominated the $\text{Ca}_v\alpha_2\delta$ expression profile in ileal PPG-cells (28). Given that the $\text{Ca}_v\alpha_2\delta$ subunit has been reported to play an important role in facilitating trafficking and cell-surface expression of the Ca_v channels, which produces increased Ca^{2+} current density, $\text{Ca}_v\alpha_2\delta\text{-1}$ involvement in GLP-1 secretion through the transport of Ca_v channels onto the plasma membrane of PPG-cells might well be expected, although further studies are needed to confirm the detailed mechanism by which $\text{Ca}_v\alpha_2\delta\text{-1}$ affects glucose-stimulated GLP-1 secretion.

Distribution of the $\text{Ca}_v\alpha_2\delta\text{-1}$ subunit is common in nervous systems, cardiac, and smooth and skeletal muscle (29). Associations between *Cacna2d1* mutations and diseases such as neuropathic pain (30), epilepsy (31), and several cardiac dysfunctions (32, 33) have been demonstrated. However, there are no reports on the association between endocrine and metabolic disorders and *Cacna2d1* mutations in human. Experiments using a cell line or knockout mice have reported involvement of $\text{Ca}_v\alpha_2\delta\text{-1}$ in the regulation of hormone secretion. In pituitary GH3 cells, $\text{Ca}_v\alpha_2\delta\text{-1}$ increase by epidermal growth factor (EGF) affects the functional expression of Ca_v channels at the plasma membrane, resulting in enhanced prolactin secretion (34). Conventional *Cacna2d1* knockout mice exhibit glucose intolerance due to insulin deficiency and increased susceptibility to diabetes (20). This study is the first to report involvement of $\text{Ca}_v\alpha_2\delta\text{-1}$ in enteroendocrine hormone secretion using intestine-specific knockout mice.

Gabapentin (GBP) is an antiepileptic and antihyperalgesic drug, which is also indicated for painful neuropathy in people with diabetes. The mechanism of action of GBP depends on its binding to the $\text{Ca}_v\alpha_2\delta$ subunit of neuronal voltage-gated calcium (Ca_v) channels to impair the ability of the $\text{Ca}_v\alpha_2\delta$ subunit to enhance Ca^{2+} influx through Ca_v channels, thus inhibiting the release of excitatory neurotransmitters (26, 35–37). Systemic administration of GBP in this study resulted in decreased GLP-1 secretion with no glucose intolerance, whereas conventional *Cacna2d1* knockout mice exhibited impaired glucose tolerance in a previous report (20). Factors behind the discrepancy between the effect of systemic defects of *Cacna2d1* gene on glucose tolerance and that of systemic administration of GBP could not be determined at this time. It would be necessary to confirm the effect of the long-term continuous use of GBP on glucose tolerance. In human, hyperglycemia is listed as a possible adverse reaction in the labeling of GBP and there is a report of hyperglycemia in people with diabetes receiving GBP (38). On the other hand, there are a few case reports of hypoglycemia associated with GBP use in people with and without

diabetes (39, 40). Thus, the effect of GBP on glucose tolerance remains equivocal in clinical use.

We found that the glucose-stimulated increase in intracellular Ca^{2+} concentration in NCI-H716 cells and GLP-1 secretion were significantly decreased by 40-h incubation with GBP (Fig. 2, C–E) with no change in cell viability (data not shown). GLP-1 content was also significantly reduced by GBP (Fig. 2B). Both reduced GLP-1 content and suppressed increase in intracellular Ca^{2+} concentration might well be involved in the reduction of GLP-1 secretion from NCI-H716 cells by GBP; the contributions of each factor remain ambiguous.

There was no significant difference in the intracellular Ca^{2+} concentration between 1 mM and 5 mM GBP loading (Fig. 2E), although GLP-1 secretion was significantly more strongly suppressed with 5 mM GBP than with 1 mM GBP (Fig. 2C). It is possible that minute differences in intracellular Ca^{2+} concentration, which could not be detected as significant differences in the present experiment, might well affect GLP-1 secretion, although we are unable to prove it in detail at this time.

RT-PCR and immunohistochemistry clearly showed the $\text{Ca}_v\alpha_2\delta\text{-1}$ expression in GLP-1-positive cells in the upper and lower small intestine (Fig. 1, B–D). On the other hand, CACNA2D1-positive/GLP-1-negative cells and CACNA2D1-negative/GLP-1-positive cells were also identified, although in very small numbers (Fig. 1E). Thus, it cannot be asserted that the expression of $\text{Ca}_v\alpha_2\delta\text{-1}$ is exclusively confined to GLP-1-producing cells. Approximately 7–8 GLP-1 (GFP)-positive or *cacna2d1*-positive cells were observed per slide, and the number of cells with distinct expression of GLP-1 and $\alpha_2\delta\text{-1}$ subunit was 1–2 per slide. The proportion of single positive cells is presumed to be low. However, we did not conduct a quantitative assessment as it is almost impossible to accurately quantify the number of cells that are originally distributed in three dimensions on a two-dimensional slide. Various recent studies have revealed that the same enteroendocrine cell can produce multiple enteroendocrine hormones (41). Subpopulations of GLP-1-producing PPG-cells coexpress cholecystokinin (CCK), secretin, GIP, peptide YY (PYY), and neurotensin (42). In fact, we identified the expression of $\text{Ca}_v\alpha_2\delta\text{-1}$ in some GIP-producing cells in the upper small intestine, but not in the lower small intestine (Fig. 1, F and G).

Consistent with the fact that channel expression was identified in some GIP-producing cells, GIP levels and GLP-1 levels were significantly lower in male CKO mice compared with their littermate controls (Fig. 4E). However, no significant differences were found between CKO and control mice with regard to blood glucose levels and insulin levels (Fig. 4, B and C); in previous reports using DKO mice, deficient GIP and GLP-1 signaling resulted in decreased insulin secretion and increased blood glucose levels (43, 44). The reasons for the lack of significant changes in blood glucose and insulin levels in the present study despite significantly reduced GIP and GLP-1 secretion in male CKO mice are unclear. However, it is possible that the number of mice ($n = 3\text{--}5$) was not sufficient to counteract the variability in measurements and that the differences in blood glucose trends could not be accurately assessed, as several mice showed blood glucose levels above the measurement sensitivity (>600 mg/dL) at 15–30 min after the OGTT.

Regarding the GLP-1 secretion in female mice, there was no significant increase in GLP-1 secretion following glucose loading (Fig. 4H). A previous report has shown that the apex of GLP-1 secretion after glucose loading in female mice is within 5 min and already declines after 10 min (45). In this study, blood samples were collected following a 15-min glucose loading period, which may not have captured the peak value of GLP-1.

In conclusion, our study provides several new findings. First, enteroendocrine PPG-cells and GIP-producing cells express functional Ca_vα₂δ-1, which has not been reported previously in enteroendocrine cells. Second, the glucose-stimulated increase in intracellular Ca²⁺ and GLP-1 secretion at 3% glucose in NCI-H716 cells were attenuated by treatment of gabapentin, an inhibitory ligand of Ca_vα₂δ-1, suggesting that Ca_vα₂δ-1 regulates glucose-stimulated GLP-1 secretion by modulating the intracellular Ca²⁺ elevation. Finally, intestine-specific *Cacna2d1* knockout mice exhibited a reduced GLP-1 secretion in response to glucose administration regardless of sex, supporting the physiological significance of Ca_vα₂δ-1 in GLP-1 secretion in vivo. These findings suggest that Ca_vα₂δ-1 may have the potential to serve as a target that affects endogenous GLP-1 secretion to facilitate the treatment of type 2 diabetes and obesity.

DATA AVAILABILITY

Data will be made available upon reasonable request.

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AUTHOR CONTRIBUTIONS

Y.Y. and S.Y. conceived and designed research; Y.Y., K.Y., and N.W. performed experiments; Y.Y. analyzed data; Y.Y., S.Y., N.H., E.I.-O., M.F., and T.M. interpreted results of experiments; Y.Y. prepared figures; Y.Y. drafted manuscript; Y.Y., S.Y., and N.I. edited and revised manuscript; Y.Y., S.Y., N.H., E.I.-O., M.F., T.M., D.Y., Y.H., and N.I. approved final version of manuscript.

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