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Sensory and motor physiological functions are impaired in gastric inhibitory polypeptide receptor-deficient mice

Tetsuji Okawa[1], Hideki Kamiya[2]*†, Tatsuhito Himeno[1], Yusuke Seino[3], Shin Tsunekawa[1], Yoshitaka Hayashi[4], Norio Harada[5], Yuichiro Yamada[6], Nobuya Inagaki[5], Yutaka Seino[7], Yutaka Oiso[1], Jiro Nakamura[1]†

Departments of [1]Endocrinology and Diabetes and [2]Chronic Kidney Disease Initiatives, Nagoya University Graduate School of Medicine, [3]Department of Metabolic Medicine, Nagoya University School of Medicine, [4]Department of Genetics, Research Institute of Environmental Medicine, Nagoya University, Nagoya, [5]Department of Diabetes and Clinical Nutrition, Kyoto University Graduate School of Medicine, Kyoto, [6]Department of Endocrinology, Diabetes and Geriatric Medicine, Akita University Graduate School of Medicine, Akita, and [7]Kansai Electric Power Hospital, Osaka, Japan

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***Correspondence**
Hideki Kamiya Tel.: +81-561-63-1683, Fax: +81-561-63-1276
E-mail: hkamiya@aichi-med-u.ac.jp

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ABSTRACT

Aims/Introduction: Gastric inhibitory polypeptide (GIP) is an incretin secreted from the gastrointestinal tract after an ingestion of nutrients, and stimulates an insulin secretion from the pancreatic islets. Additionally, GIP has important roles in extrapancreatic tissues: fat accumulation in adipose tissue, neuroprotective effects in the central nervous system and an inhibition of bone resorption. In the current study, we investigated the effects of GIP signaling on the peripheral nervous system (PNS).

Materials and Methods: First, the presence of the GIP receptor (GIPR) in mouse dorsal root ganglion (DRG) was evaluated utilizing immunohistochemical analysis, western blotting and reverse transcription polymerase chain reaction. DRG neurons of male wild-type mice (WT) were cultured with or without GIP, and their neurite lengths were quantified. Functions of the PNS were evaluated in GIPR-deficient mice (*gipr*–/–) and WT by using current perception thresholds (CPTs), Thermal Plantar Test (TPT), and motor (MNCV) and sensory nerve conduction velocity (SNCV, respectively). Sciatic nerve blood flow (SNBF) and plantar skin blood flow (PSBF) were also evaluated.

Results: We confirmed the expression of GIPR in DRG neurons. The neurite outgrowths of DRG neurons were promoted by the GIP administrations. The *gipr*–/– showed impaired perception functions in the examination of CPTs and TPT. Both MNCV and SNCV were delayed in *gipr*–/– compared with these in WT. There was no difference in SNBF and PSBF between WT and *gipr*–/–.

Conclusions: Our findings show that the GIP signal could exert direct physiological roles in the PNS, which might be directly exerted on the PNS.

INTRODUCTION

Gastric inhibitory polypeptide (GIP) is one of the gastrointestinal regulatory peptides synthesized by K cells of the duodenum and small intestine[1]. GIP potentiates meal-induced insulin secretion and lower blood glucose level[1]. Recently, incretin-based therapies have been used clinically as novel therapy for type 2 diabetes, using receptor agonists of glucagon-like peptide-1 (GLP-1), another incretin, and inhibitors of the incretin-degrading enzyme dipeptidyl peptidase-4 (DPP-4)[2–4]. Although both GLP-1 receptor (GLP-1R) agonists and DPP-4 inhibitors (DPP-4I) improve glycemic control in type 2 diabetes patients, there is no consensus regarding the antidiabetic effect for GIP receptor (GIPR) agonists[5–7]. In addition, the extrapancreatic physiological function of GIP, the increase of lipoprotein lipase activity and fat accumulation, might cause the delay of clinical application of GIPR agonists[8]. Furthermore, there are some

†Present address: Division of Diabetes, Department of Internal Medicine, Aichi Medical University School of Medicine, Nagakute, Japan.
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reports of other extra-islet functions of GIP: inhibition of bone resorption[9], decrease of intestinal motility[10], and neurotrophic effects in the central nervous system (CNS)[11–13]. Some of these functions could be beneficial for type 2 diabetes patients frequently complicated by osteoporosis[14] and cognitive disorder[15]. Therefore, GIPR agonists should be considered as an independent therapeutic tool for type 2 diabetes treatment.

Recent studies have described the important roles of some intestinal peptides in nerve development, regeneration and neuronal survival[16,17]. Many reports have suggested that GLP-1R agonists have neuroprotective properties in both the CNS[18,19] and the peripheral nervous system (PNS)[20–22]. The expressions of GIP and GIPR have been reported in the large pyra-midal neurons in the cortex and the hippocampus[12,13]. One of these reports also showed that the proliferation of neuronal progenitors was enhanced by exogenous GIP, and was decreased in the dentate gyrus of GIPR-deficient mice (*gipr*–/–)[12]. In another study, it has been reported that protease-resistant GIP facilitated hippocampal long-term potential (LTP) and improved impaired LTP induced by beta-amyloid[11]. In contrast to the CNS, there are few studies that evaluate the physiological function of GIP/GIPR signaling in the PNS[23]. Buhren *et al.*[23] showed that axonal regenerations were impaired in the *gipr*–/– compared with wild-type mice (WT) after crush injuries of sciatic nerves. With regard to DPP-4I (vildagliptin), prevention of peripheral nerve degeneration in streptozotocin-induced diabetic rats has recently been shown[24]. Although active GIP is certainly increased by DPP-4I, many other bioactive peptides, such as neuropeptide Y (NPY), substance P (SP), GLP-1, glucagon-like peptide-2 and stromal cell–derived factor-1α (SDF-1α), have also been reported as substrates of DPP-4[25]. Thus, the preventive effects of DPP-4I on diabetic polyneuropathy (DPN) might be mediated through increased levels of GIP, but is attributed to these other peptides. Although we have already reported the beneficial effects of GLP-1R agonist on DPN[20], the effects of GIP on peripheral nerve functions have not yet been evaluated. Therefore, in the present study, we focused on the direct physiological roles of GIP/GIPR signaling in undamaged PNS, and assessed the neurological dysfunction of GIPR-deficient mice (*gipr*–/–).

MATERIALS AND METHODS

Primary Culture of Dorsal Root Ganglion Neurons

Dorsal root ganglion (DRG) neuron cultures were prepared from 5-week-old male C57BL/6 mice (Chubu Kagaku Shizai, Nagoya, Japan) and GIPR-deficient mice as previously described[26]. The collected DRG were incubated in 0.12% collagenase (Wako Pure Chemical, Osaka, Japan) and dissociated using a flame-narrowed glass pipette. DRGs were diluted in a medium consisting of F-12 media supplemented with 30 nmol/L selenium and seeded on glass cover slips coated with poly-L-lysine.

Evaluation of Neurite Outgrowth

DRG neurons cultured for 24 h with or without human GIP (Peptide Institute, Osaka, Japan) were fixed with 4% paraformaldehyde (PFA) and incubated with rabbit polyclonal anti-neurofilament heavy-chain antibody (1:5000; Millipore, Billerica, MA, USA), followed by Alexa Fluor 594-coupled goat anti-rabbit immunoglobulin G (IgG) antibody (1:200; Invitrogen, Tokyo, Japan). Neurite outgrowths were analyzed in 10 neurons per cover slip.

Reverse Transcription Polymerase Chain Reaction

Ribonucleaic acids (RNAs) were extracted from frozen samples of DRGs and the pancreas using Isogen (Nippon Gene, Toyama, Japan). RNAs were reverse transcribed and real-time polymerase chain reaction (PCR) was carried out utilizing the Mx3000P QPCR System (Stratagene Agilent Technologies, Santa Clara, CA, USA) using SYBR Green I (Applied Biosystem, Foster City, CA, USA). Primer sequences are as follows. GIP-R, (f) GGATCTTGGAGAGACCACACTC, (r) TAAGA TGAGTAGGGCTAGCAGCAG; β-actin, (f) CATCCGTAAAG ACCTCTATGCCAAC (r) ATGGAGCCACCGATCCACA. The PCR products were analyzed by agarose gel/ethidium bromide to confirm these predicted lengths.

Western Blotting

Samples were lysed in detergent lysis buffer (Cell Lysis Buffer; Cell Signaling Technology, Boston, MA, USA). The concentrations of proteins were quantitated with a bicinchoninic acid assay (Sigma Chemical, St Louis, MO, USA), and were transferred to polyvinylidene fluoride membranes (Millipore) after sodium dodecyl sulfate polyacrylamide gel electrophoresis. The membranes were incubated with goat polyclonal anti-GIPR antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal anti-β-actin antibody (1:10,000; Abcam, Cambridge, MA, USA). The antigen detection was carried out using ECL Plus Reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with horseradish peroxydase-conjugated anti-goat or rabbit IgG antibody (1:6,000; Cell Signaling Technology).

Animals

The generation and characterization of *gipr*–/– has been described previously[8]. The male *gipr*–/– and male wild-type C57BL6/J mice (WT; Chubu Kagaku Shizai) were housed in an aseptic room with a 12-h light cycle and fed *ad libitum*. Both WT and *gipr*–/– at 21-weeks-old were used for measurement of current perception thresholds, Thermal Plantar Test, motor and sensory nerve conduction velocity, sciatic nerve blood flow, plantar skin blood flow, and immunohistochemistry. The Nagoya University Institutional Animal Care and Use Committee approved the protocols of this experiment.

Measurement of Current Perception Threshold

To evaluate the plantar sensory perception, current perception thresholds (CPT) were measured in both WT and *gipr*–/– using a CPT/LAB Neurometer (Neurotron, Denver, CO, USA). Two electrodes for stimulation were attached to plantar surfaces

of a mouse kept in a Ballman cage (Natsume Seisakusho, Tokyo, Japan). Transcutaneous electric stimuli with three different frequencies (2,000, 250 and 5 Hz) were applied to the plantar surfaces. The intensity of stimulation was gradually increased. The minimum intensity at which a mouse withdrew its paw was defined as the CPT. Six consecutive measurements were carried out at each frequency.

Thermal Plantar Test

Paw withdrawal response to thermal stimuli of radiant heat was measured using a device (Plantar Test, 7370; Ugo Basile, Comerio, Italy). The paw withdrawal latencies were measured five times per session, separated by a minimum interval of 10 min. Paw withdrawals as a result of locomotion or weight shifting were not counted.

Nerve Conduction Velocity

The anesthetized mice were placed on a heated pad to ensure a constant rectal temperature of 37°C. Motor nerve conduction velocity (MNCV) was determined between a sciatic notch and an ankle as previously described[27,28]. The sensory NCV (SNCV) was measured between a knee and an ankle with retrograde stimulation.

Sciatic Nerve Blood Flow and Plantar Skin Blood Flow

Sciatic nerve blood flow (SNBF) and plantar skin blood flow (PSBF) were measured by laser Doppler flowmetry (FLO-N1; Omega Wave Inc, Tokyo, Japan) as previously described[20]. The sciatic nerves were exposed and the blood flows were measured by a probe placed 1 mm above the nerve. To determine PSBF, three different spots of plantar skin were selected to be measured. During this measurement, the mouse was placed on a heated pad.

Tissue Collection and Immunohistochemistry

Dissected pancreas and DRGs were fixed in 4% PFA, immersed in phosphate-buffered saline containing 20% sucrose, embedded and cut into 5-μm sections. Sections were blocked with 5% skim milk (Meiji Milk, Tokyo, Japan), and were applied with the goat polyclonal anti-GIPR antibody (1:100; Santa Cruz Biotechnology), followed by the Alexa Fluor 594-coupled donkey anti-goat IgG antibody (1:200; Invitrogen). Nucleus staining was carried out using 4′,6-diamidino-2-phenylindole (Merck).

Statistical Analysis

All the group values are expressed as means ± standard deviation. Statistical analyses were made by one-way ANOVA, with the Bonferroni correction for multiple comparisons. All analyses were carried out by personnel unaware of the animal identities.

RESULTS

DRG Neurons Expressed GIPR

To confirm the quality of the GIPR antibody obtained from Santa Cruz Biotechnology, we compared the immunostaining of the islets of *gipr*–/– and WT. The antibody detected the GIPR protein in the islets of WT, but not in those of *gipr*–/– (Figure 1a). Using this antibody, GIPR proteins were detected in DRG neurons of WT, but not in those of *gipr*–/– (Figure 1b). The expressions of GIPR were observed in all sizes of neurons, and also in satellite glias. In addition to immunohistochemistry, GIPR proteins in the DRGs of WT were detected by western blot (WB) analysis, and those of *gipr*–/– were undetected by WB analysis (Figure 1c). GIPR messenger RNA in the pancreas and DRGs of WT were detected by reverse transcription (RT)–PCR (Figure 1d).

GIP Promoted Neurite Outgrowth of DRG Neurons

It has been reported that axonal regrowth was impaired in *gipr*–/– after a sciatic nerve crush injury. Therefore, we used DRG culture system to evaluate the impact of the GIP on the PNS, especially sensory neurons. In our culture condition, only large neurons elongated their neurites, and the neurite outgrowths were promoted by the addition of GIP (Figure 2a). Joint numbers of the neurites were increased by GIP (control 25.2 ± 5.80/cell GIP 10 nmol/L; 82.2 ± 8.87, GIP 100 nmol/L; 91.8 ± 4.08, GIP 1,000 nmol/L; 113.8 ± 12.77 control vs GIP 10 nmol/L, $P < 0.05$; GIP 10 nmol/L vs GIP 100 nmol/L, $P < 0.05$; GIP 100 nmol/L vs GIP 1,000 nmol/L, $P < 0.05$; Figure 2b). In addition, total lengths of the neurites were significantly increased in all GIP-loaded groups (control 430.0 ± 40.85 μm/cell, GIP 10 nmol/L; 901.0 ± 31.83, GIP 100 nmol/L; 1067.0 ± 85.12 GIP 1,000 nmol/L; 1667.4 ± 77.89 control vs GIP 10 nmol/L, $P < 0.05$; GIP 10 nmol/L vs GIP 100 nmol/L, $P < 0.05$; GIP 100 nmol/L vs GIP 1,000 nmol/L, $P < 0.05$; Figure 2b). Neurite outgrowths were not promoted in DRG neurons of GIPR-deficient mice (joint number: control 25.1 ± 4.43/cell, GIP 1,000 nmol/L; 26.7 ± 2.49; $P = 0.33$, total length: control 462.1 ± 34.07 μm/cell, GIP 1,000 nmol/L; 452.5 ± 31.14; $P = 0.51$; Figure 2c).

Bodyweights and Blood Glucose Levels

Random blood glucose levels and bodyweight measured during the experimental period were not significantly changed between WT and *gipr*–/– (Table 1), consistent with the previous report[8].

Sensory Perceptions Were Impaired and NCVs Were Decreased in the *gipr*–/–

We evaluated sensory functions using CPTs. In *gipr*–/–, all three CPTs were significantly increased compared with those in WT, representing hypoalgesia (Figure 3a–c). In the examination of CPTs, each electric pulse at 2,000, 250 and 5 Hz mainly stimulates large myelinated(Aβ-), small myelinated (Aδ-), and small unmyelinated (C-) fibers, respectively[29]. However, these stimuli are not actual stimuli. Therefore, we reconfirmed the impaired sensory functions using the thermal plantar test (TPT). The delays of withdrawal response times were observed in *gipr*–/– compared with WT (Figure 3d),

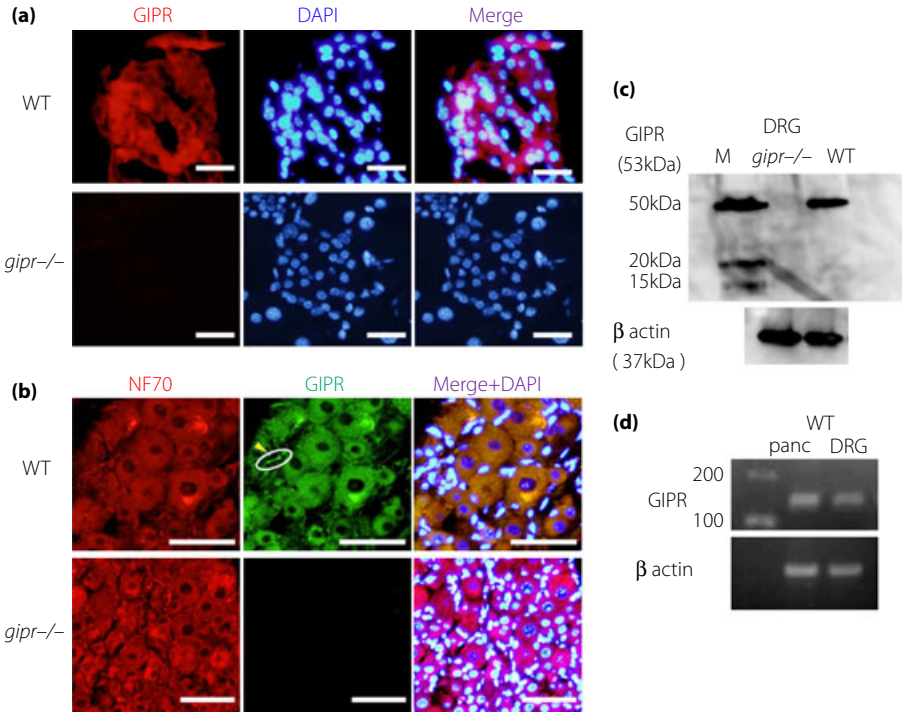

Figure 1 | Expressions of gastric inhibitory polypeptide receptor (GIPR) in dorsal root ganglions (DRGs) and the pancreas. (a) GIPR proteins (red) were detected by the anti-GIPR antibody in pancreatic islets of wild-type mice (WT), but not in those of GIPR-deficient mice (*gipr–/–*). Nuclei were stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI; blue). Scale bars, 50 μm. (b) GIPR proteins (green) were detected in DRG in WT. The expressions were detected in DRG neurons shown by NF70 antibody (red) and satellite glias (yellow arrowheads). Scale bars, 50 μm. (c) GIPR proteins in DRGs were detected with expected molecular weight in WT, but not in *gipr–/–* by western blot analysis. (d) The expressions of GIPR were confirmed in DRG neurons and pancreas by reverse transcription polymerase chain reaction. M, molecular markers; NF, neurofilament.

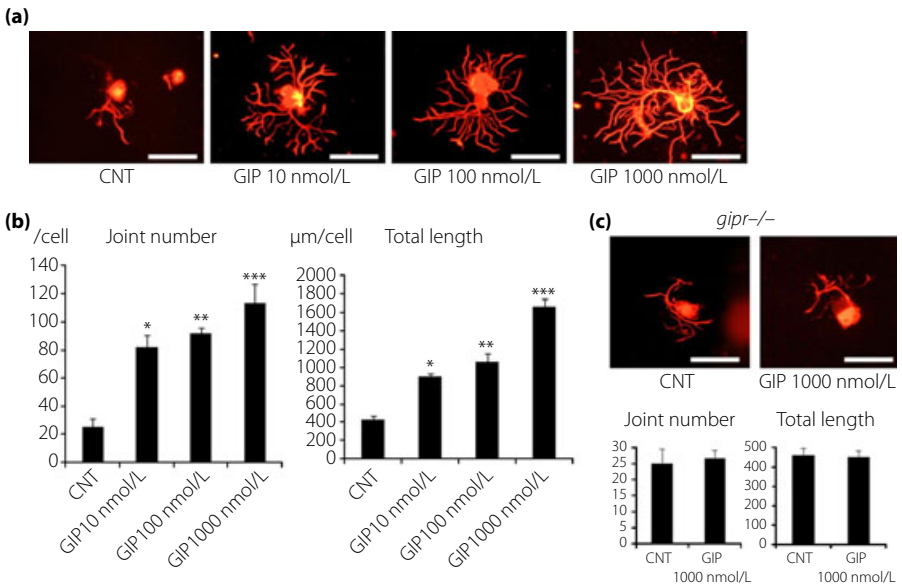

Figure 2 | Neurite outgrowths of dorsal root ganglion (DRG) neurons. (a) Representative fluorescence micrographs of DRG neurons cultured in the absence or presence of gastric inhibitory polypeptide (GIP). Scale bars, 50 μm. (b) GIP significantly promoted total neurite length and increased joint number of neurites in a dose-dependent manner. (c) GIP did not promote neurite outgrowth of DRG neurons in *gipr–/–*. Results are means ± standard deviation. Control vs GIP 10 nmol/L, $^{*}P < 0.05$. GIP 10 nmol/L vs GIP 100 nmol/L, $^{**}P < 0.05$. GIP 100 nmol/L vs GIP 1,000 nmol/L, $^{***}P < 0.05$. CNT; F-12 control media.

Table 1 | Bodyweights and blood glucose levels

	WT (n = 8)	*gipr*–/– (n = 8)
Blood glucose (mmol/L)	10.7 ± 0.3	**11.4 ± 1.2
Bodyweight (g)	33.0 ± 1.1	*29.4 ± 2.5

gipr–/–, gastric inhibitory polypeptide receptor deficient mice; WT, wild-type mice. Results are means ± standard deviation. **P = 0.35 vs wild-type mice (WT). *P = 0.52 vs WT.

suggesting a significant reduction of thermal sensitivity or thermal nociception.

The MNCVs and the SNCVs of *gipr*–/– were decreased significantly compared with those of WT (Figure 3e–f).

There was No Significant Aberration in the Peripheral Blood Flows of *gipr*–/–

As neurophysiological functions are influenced by a hemodynamic status, we examined the blood flows, SNBF and PSBF, using the laser Doppler measurement. The SNBF and PSBF in *gipr*–/– were comparable with those in WT (SNBF: WT 20.2 ± 1.73 mL/min/100 g; *gipr*–/–: 19.0 ± 2.49, P = 0.20; PSBF: WT 24.5 ± 1.98; *gipr*–/–: 22.7 ± 2.78, P = 0.09; Figure 4).

DISCUSSION

In the current study, we investigated whether the GIP/GIPR signal has some effects that maintain intact functions of the PNS in mice. First, we reconfirmed the expression of GIPR on DRG neurons using immunohistochemistry (IHC), WB and RT–PCR. Second, we showed that GIP promotes neurite outgrowths in the cultures of DRG neurons. Third, we showed that the sensory functions are reduced and NCVs are delayed in hindlimbs of *gipr*–/–. Finally, we confirmed that there is no difference in peripheral blood flow between WT and *gipr*–/–. These results show that GIP has direct beneficial effects on the PNS.

Although the expressions of GIP and GIPR in the CNS have been reported and proven[12, 13], proof of the expressions in the PNS are still insufficient[23]. In the present study, we confirmed expression of GIPR in DRG neurons using the GIPR antibody,

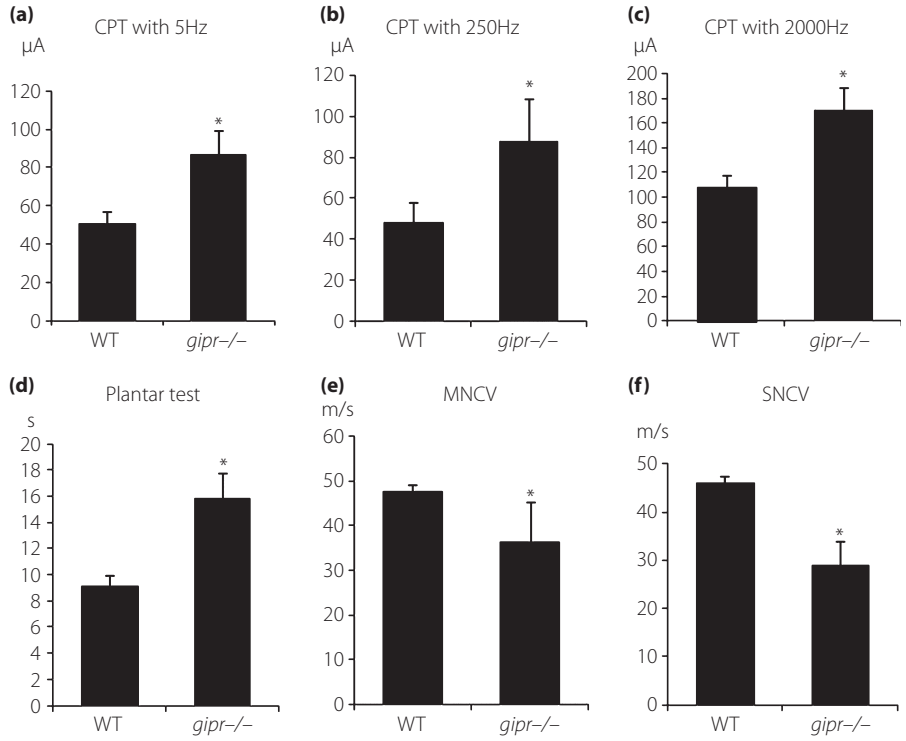

Figure 3 | Functions of the peripheral nervous system. Measurements of current perception thresholds at (a) 5, (b) 250 and (c) 2,000 Hz by Neurometer were carried out. All current perception thresholds (CPTs) were significantly increased in the gastric inhibitory polypeptide receptor deficient mice (*gipr*–/–) compared with those of wild-type mice (5 Hz: WT 50.7 ± 6.07 μA, *gipr*–/– 87.1 ± 12.53; 250 Hz: WT 48.5 ± 9.88, *gipr*–/– 87.8 ± 21.18; 2,000 Hz: WT 108.5 ± 8.99, *gipr*–/– 170.0 ± 19.14; 5 Hz: WT vs *gipr*–/–, *P < 0.0001; 250 Hz: WT vs *gipr*–/–, *P < 0.0001; 2,000 Hz: WT vs *gipr*–/–, *P < 0.0001). (d) The withdrawal response times using Thermal Plantar Test (TPT) were delayed in *gipr*–/– compared with those in WT. *gipr*–/–: GIP receptor deficient mice. The (e) motor nerve conduction velocities (MNCVs) and (f) sensory nerve conduction velocities (SNCV) of *gipr*–/– were significantly delayed compared with those of normal mice (MNCVs: WT 47.7 ± 1.49 m/s, *gipr*–/– 36.4 ± 9.17, *P < 0.0001; SNCVs: WT 46.2 ± 1.38, *gipr*–/– 29.0 ± 5.17, *P < 0.0001; n = 8 in each group).

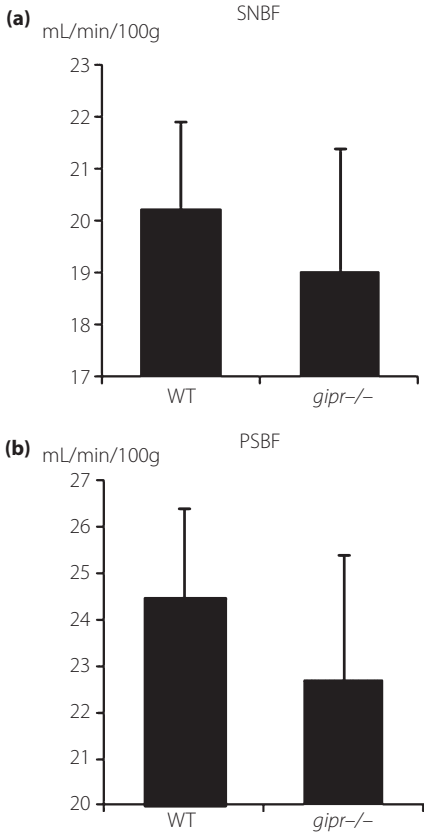

Figure 4 | Sciatic nerve blood flow (SNBF) and plantar skin blood flow (PSBF). The SNBF and PSBF in gastric inhibitory polypeptide receptor deficient mice (*gipr*–/–) mice were comparable with those in wild-type mice (WT). Results are means ± standard deviation (SNBF: WT 20.2 ± 1.73 mL/min/100 g, *gipr*–/– 19.0 ± 2.49, $P = 0.20$; PSBF: WT 24.5 ± 1.98, *gipr*–/– 22.7 ± 2.78, $P = 0.09$; $n = 8$ in each group).

the adequacy of which was assessed by comparison with positive and negative control staining. This result is consistent with the previous study in which GIPR was found to be expressed in DRG neurons and satellite glia[23].

Promotion of axonal regrowth by GIP has been described in nerve-injured model animals[23], it was still unclear whether GIP had a direct impact on the PNS or whether GIP exerted its potential through systemic effects. Therefore, we tried to evaluate the beneficial effect of GIP on axonal growth using *in vitro* DRG cultures. The neurite outgrowth was promoted dose-dependently by GIP. This result shows that GIP might have direct effects on the PNS. However, there were some limitations in our DRG culture system. First, many different types of cells were contained in the culture: neurons, satellite glia, fibroblasts and hematocytes. As a result, we could not conclude whether the effects of GIP were produced on neurons directly or indirectly through other types of cells. Second, only large sized neurons elongated their neurites in our culture. To minimize the influences of other biologically active substances, we refrained from the use of commercially available supplements or media for neuron cultures in our medium. Furthermore, the medium was tested many times to ascertain that neurons could survive in the F-12 medium supplemented only by selenite. Unfortunately, although the neurons survived, only large neurons formed neurites in this medium. Therefore, our obtained data must be considered as inconclusive evidence and the medium needs to be additionally modified in the future studies.

To investigate the physiological role of the GIP/GIPR signal on the PNS, we used the *gipr*–/–. We evaluated sensory nerve functions through the use of CPTs. The CPT measurement is clinically used to examine peripheral nerve functions in various neuropathies[30]. In the present study, reduced responsiveness against each electrical stimulation was observed in the *gipr*–/–. These results represented multiple perception impairments. Additionally, we reconfirmed a part of the dysfunction using another test, the TPT. We evaluated the NCV of lower limbs, which is the most established method ascertaining dysfunction of the PNS. Both the MNCVs and SNCVs were delayed in the *gipr*–/–. The decrease of MNCV was consistent with previous data that found the GIP and GIPR proteins in spinal motor neurons[23]. However, the outcome of reduced NCVs should be interpreted carefully. Because we have limited data to explain the phenomenon, the functional impairment needs to be examined through both pathological and intercellular molecular biological aspects in the future. Additionally, as evaluation of structural changes on the PNS in the *gipr*–/– has not yet been carried out, the question remains whether these deficits in the *gipr*–/– could be comparable with those in other diabetic animal models or humans. We also consider the probability that maturational retardation might influence the development of the neuropathic phenotype in this model, although no maturational retardation in the *gipr*–/– has been shown up to the present. To resolve these questions, further experiments including sequential morphological examinations of the PNS should be carried out in the future.

Decreased nerve blood flow has been recognized as an important factor in the development of DPN. Although there is no report about the influence of GIP on hemodynamics, we examined the nerve and skin blood flow to exclude influence on functions of the PNS. As expected, the amounts of these blood flows in *gipr*–/– were equivalent to those in WT.

In conclusion, although these data might suggest important physiological roles of GIP/GIPR signals on the PNS, further intervention studies are required to ascertain the effect of incretin-based drugs on DPN.

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