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ORIGINAL ARTICLE

Hypothalamic Brain-Derived Neurotrophic Factor Regulates Glucagon Secretion Mediated by Pancreatic Efferent Nerves

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Understanding the molecular mechanism of the regulation of glucagon secretion is critical for treating the dysfunction of α cells observed in diabetes. Glucagon-like peptide (GLP)-1 analogues reduce plasma glucagon and are assumed to contribute to their action to lower blood glucose. It has previously been demonstrated that the central administration of brain-derived neurotrophic factor (BDNF) improves glucose metabolism by a mechanism independent of feeding behaviour in obese subjects. Using male rats, we examined whether BDNF influences glucagon secretion from α cells via the the central nervous system. We investigate whether: (i) the central infusion of BDNF stimulates glucagon and/or insulin secretion via the pancreatic efferent nerve from the hypothalamus; (ii) the intraportal infusion of GLP-1 regulates glucose metabolism via the central and peripheral nervous system; and (iii) BDNF receptor and/or BDNF-positive fibres are localised near α cells of islets. The portal glucagon level decreased with the central administration of BDNF ($n = 6$, in each; $P < 0.05$); in contrast, there was no significant change in portal insulin, peripheral glucagon and insulin levels with the same treatment. This reduction of glucagon secretion was abolished by pancreatic efferent denervation ($n = 6$, in each; $P < 0.05$). In an immunohistochemical study, pancreatic α cells were stained specifically with BDNF and tyrosine-related kinase B, a specific receptor for BDNF, and α cells were also co-localised with BDNF. Moreover, intraportal administration of GLP-1 decreased glucagon secretion, as well as blood glucose, whereas it increased the BDNF content in the pancreas; these effects were inhibited with the central infusion of BDNF antibody ($n = 6$, in each; $P < 0.05$). BDNF and GLP-1 affect glucose metabolism and modulate glucagon secretion from pancreatic α cells via the central and peripheral nervous systems.

Key words: brain-derived neurotrophic factor, GLP-1, hypothalamus, glucagon, pancreatic efferent nerve

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Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophic factor family, which plays a key role in regulating the survival, growth and maintenance of neurones (1). BDNF reduces food intake and lowers blood glucose levels in obese diabetic mice (2–4). In animals with conditional BDNF deletion, mutation or, in BDNF (+/−) heterozygous mice, hyperphagia and obesity, are accompanied by significantly reduced BDNF gene expression in the hypothalamus, including the ventromedial nucleus of the hypothalamus (VMH) (5). Infusion of BDNF into the brains of diabetic mice improved their peripheral insulin sensitivity, apparently as result of a mechanism independent of a change in food intake (6). However, when BDNF is administered to normal mice or rats, it has no effects on blood glucose levels, indicating that BDNF exerts its effects by enhancing insulin sensitivity (2).

Although the mechanism by which BDNF signalling in the brain affects peripheral glucose metabolism remains to be determined, BDNF may also exert direct actions on peripheral tissues involved in energy metabolism, including muscles and the pancreas (7, 8). One possible mechanism by which this might occur involves modulation of the autonomic nervous system. It is, however, doubtful whether the effect of BDNF on blood glucose levels is accounted for only by its effects on the hypothalamus because tyrosine-related kinase B (TrkB) is found not only in the central and peripheral nervous system, but also in some non-neuronal tissues, including rat and

human pancreatic α cells (9). During hypoglycaemia, the VMH in the hypothalamus plays a critical role in stimulating the release of glucagon, suggesting that the central nervous system may play an important role in regulating pancreatic α cell glucagon secretion (10). Our previous study suggests that the rise in portal glucagon-like peptide (GLP)-1 levels acts in the hypothalamus via the hepatic vagal afferent nerve to regulate food intake and that different neural mechanisms mediate the regulation of feeding behaviour and glucose metabolism (11). Thus, we investigated whether: (i) the central administration of BDNF affects glucagon/insulin secretion; (ii) efferent denervation to the pancreas affects BDNF-induced alterations of glucagon/insulin secretion; (iii) portal administration of GLP-1 affects the BDNF content in hypothalamus and pancreas; and (iv) the central administration of BDNF-antibody affects GLP-1-induced alterations of pancreatic BDNF content.

Materials and methods

Animals

Male Sprague–Dawley rats (250–280 *g*; Seac Yoshitomi, Fukuoka, Japan) were housed in a room under a 12/12 h light/dark cycle (lights on 07.00 h) and maintained at 21 ± 1 °C and 55 ± 5% humidity. Animals were permitted *ad lib.* access to standard chow (Clea chow, Clea, Japan) and tap water. All rats were handled for 5 min each on four successive days to habituate their arousal levels before the experiment. On the testing day (approximately 7 days after the treatment), the animals had recovered to at least their pre-treatment body weight. All studies were conducted in accordance with the Oita University Guidelines, which are based on the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Surgery

Rats were placed in a stereotaxic apparatus under anaesthesia (50 mg/kg sodium pentobarbital, i.p.; Narishige, Tokyo, Japan). A stainless steel guide cannula (23 gauge) was chronically implanted into the third cerebroventricle (3vt). This procedure was performed at least 10 days before the start of infusions. A stainless steel wire stylet (30 gauge) was inserted into the guide cannula to prevent cerebrospinal fluid leakage and cannula obstruction. A stainless steel cannula (23 gauge; 15 mm long) was inserted into the 3vt along the midline, 6.0 mm anterior to the zero ear bar coordinate, to a depth of 7.8 mm from the cortical surface according to the atlas of Paxinos and Watson (12). Small catheters (Intramedic PE-10; Clay Adams, Parsippany, NJ, USA) were inserted into the portal vein for blood sampling or infusion. The portal line was initiated in the superior mesenteric vein and positioned with the tip approximately 1.0–1.5 cm from the porta hepatis. The catheters were maintained with a daily flush of 10–20 μl of heparinised bacteriostatic saline for the first 10 days after the operation, and then every 3 days to ensure the patency. For pancreatic efferent denervation, the abdominal cavity was opened and pancreatic branch of the vagal (VPN) and the splanchnic nerves (SPN) were cut in the region where they enter the pancreas. For sham operation, the abdomen was opened, although the VPN and SPN were not cut (13). On the testing day, the animals had recovered to at least body weight before the operation.

Reagents

On the day of the experiment, BDNF (0.1 μg/μl; Sigma, St Louis, MO, USA), anti-BDNF antibody (for neutralisation and immunohistochemistry, 0.1 μg/μl; Millipore, Billerica, MA, USA) and GLP-1 (10 pmol/mL; Sigma) were freshly dissolved in phosphate-buffered saline (PBS). The pH of each solution was adjusted to 6.5–7.5.

Experimental protocol

In design 1, rats were assigned to one of two different groups (n = 6 in each group). In group 1, PBS (10 μl/10 min, i.c.v.) was administered. In group 2, BDNF (1 μg/10 μl/10 min, i.c.v.) was administered.

In design 2, rats were assigned to one of four different groups (n = 6 in each group). In group 1, PBS (10 μl/10 min, i.c.v.) was administered 2 weeks after sham operation. In group 2, BDNF (1 μg/10 μl/10 min, i.c.v.) was administered 2 weeks after sham operation. In group 3, PBS (10 μl/10 min, i.c.v.) was administered 2 weeks after pancreatic efferent denervation. In group 4, BDNF (1 μg/10 μl/10 min, i.c.v.) was administered 2 weeks after pancreatic efferent denervation.

In design 3, rats were assigned to one of four different groups (n = 6 in each group). In group 1, PBS (10 μl/10 min, i.c.v.) was administered 2 h before PBS treatment (100 μl/10 min, intraportally). In group 2, anti-BDNF antibody (1 μg/10 μl/10 min, i.c.v.) was administered 2 h before PBS treatment (10 μl/10 min, intraportally). In group 3, PBS treatment (10 μl/10 min, i.c.v.) was administered 2 h before GLP-1 (1 pmol/100 μl/10 min, intraportally). In group 4, anti-BDNF antibody (1 μg/10 μl/10 min, i.c.v.) was administered 2 h before GLP-1 (1 pmol/100 μl/10 min, intraportally).

Measurements of glucagon, insulin, BDNF and GLP-1 levels

After all rats were anaesthetised with sodium pentobarbital (50 mg/kg, i.p.), blood samples were collected from the tail vein and portal vein. Blood from all rats was centrifuged at 4000 **g** for 10 min at 4 °C. They were exsanguinated following transcardiac perfusion with 100 ml of saline containing 200 U of heparin. Pancreas tissues were homogenised and extracted with acid–ethanol solution (concentration HCl : ethanol : H_2O = 1.5 : 75 : 23.5) and insulin and glucagon concentrations in the supernatant were measured. Plasma and homogenate were immediately frozen and stored at −80 °C until analysed. The pancreatic and plasma glucagon concentrations were measured by radioimmunoassay (Phoenix Pharmaceutical Inc., CA, USA), the pancreatic and plasma insulin concentrations were measured by an insulin enzyme-linked immunosorbent assay (ELISA) (Shibayagi, Gunma, Japan), and the pancreatic and plasma mature form of BDNF concentrations were measured with a BDNF ELISA (Insight Genomics, Falls Church, VA, USA). The homogenate was boiled for 10 min, and a 50-μl aliquot was removed for protein assay (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry

Rats were anaesthetised with 50 mg/kg sodium pentobarbital i.p., and then transcardially perfused with 50 ml PBS containing 50 U heparin, followed by 50 ml of 4% *p*-formaldehyde in ice-cold PBS. The brains and pancreas were removed and rapidly frozen at −80 °C and stained for BDNF, TrkB, glucagon, insulin and *c-fos*. The brains for *c-fos* staining were taken 2 h after treatment of PBS or GLP-1. Frozen sections were cut from hypothalami at a thickness of 5 μm using a cryostat at −20 °C. The sections were incubated overnight at 4 °C with polyclonal rabbit anti-rat insulin (dilution 1 : 100; Cell Signaling Technology Inc., Danvers, MA, USA), rabbit anti-rat glucagon (dilution 1 : 100; Cell Signaling Technology Inc.), rabbit anti-rat TrkB (dilution 1 : 100; Novus Biologicals, Litteton, CO, USA) and rabbit anti-rat BDNF (dilution 1 : 100; Abcam, Cambridge, MA, USA), followed by detection with biotin-conjugated goat anti-rabbit immunoglobulin G (ABC

reagent; Vector Laboratories, Burlingame, CA, USA). The immunoreactivity for *c-fos* was visualised with 3,3′-diaminobenzidine (DAB) (Nacalai Tesque, Kyoto, Japan). BDNF was visualised with DAB or rhodamine-conjugated streptavidin (ABC reagent; Vector Laboratories). Glucagon and insulin were visualised with fluorescein isothiocyanate-conjugated streptavidin (ABC reagent; Vector Laboratories) and TrkB was visualised with rhodamine-conjugated streptavidin (ABC reagent; Vector Laboratories). Normal rabbit serum was used in place of the aforementioned antibodies as a negative control.

Glucose tolerance test

After an overnight fast, rats were injected i.p. with glucose (2.0 g/kg body weight) and blood samples were taken at 0, 15, 30, 60 and 120 min. Blood glucose concentrations were measured using the glucose oxidase method and a glucose analyser (MS-GR101; Terumo, Tokyo, Japan). Serum insulin concentrations were determined using an insulin ELISA (Shibayagi).

Statistical analysis

The data are expressed as the mean ± SEM. Statistical significance was evaluated using a two-way ANOVA followed by Scheffe's test for post-hoc comparisons. For all tests, $P < 0.05$ was considered statistically significant.

Results

Effect of central BDNF administration on plasma glucagon, insulin level, and plasma and pancreatic BDNF levels

Intracerebroventricular administration of BDNF significantly decreased portal but not peripheral glucagon levels compared to PBS treatment ($n = 6$ per group, $P < 0.05$) although the i.c.v. administration of BDNF did not affect portal or peripheral insulin levels (Fig. 1A–D). In addition, there was no significant difference in either portal and peripheral GLP-1 levels between the BDNF and PBS treatments. The central infusion of BDNF elevated pancreatic BDNF contents compared to PBS treatment, although it did not alter plasma BDNF levels, or portal and peripheral GLP-1 levels (Fig. 1F–I).

Immunohistochemical staining for TrkB and BDNF in the pancreas

Immunohistochemical analysis of double-labelling demonstrated that Trk-B-like immunoreactivity is co-localised with glucagon-like immunoreactivity (Fig. 2A) but not insulin-like immunoreactivity (Fig. 2B) in the pancreas. BDNF-positive fibres were observed in peripheral areas

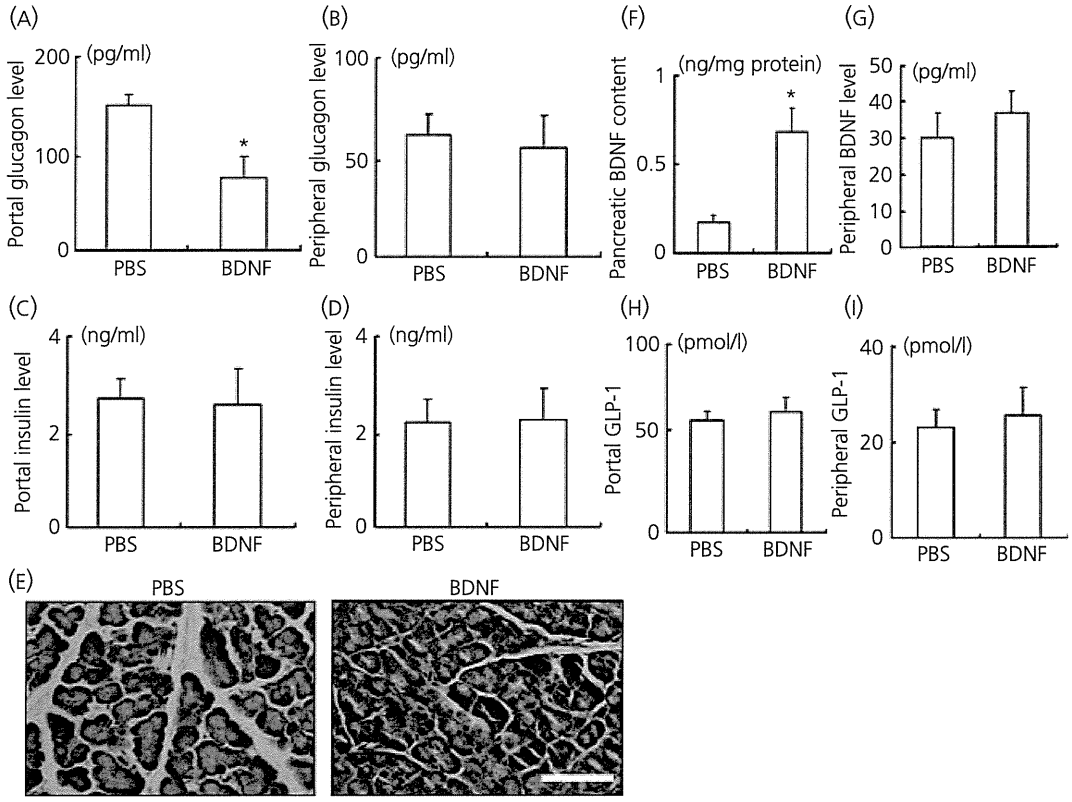

Fig. 1. I.c.v. administration of brain-derived neurotrophic factor (BDNF) reduces the portal glucagon level and increases pancreatic BDNF content. Portal (A) and peripheral (B) glucagon levels of each group ($n = 6$). Portal (C) and peripheral (D) insulin levels of each group ($n = 6$). (E) Representative immunostaining for BDNF staining performed with pancreas sections derived from each group. Pancreatic (F) and peripheral (G) BDNF levels of each group ($n = 6$). Portal (H) and peripheral (I) GLP-1 levels of each group ($n = 6$). *$P < 0.05$ versus phosphate-buffered saline (PBS) group. Scale bar = 100 μm. Treatment groups: PBS, intraportal administration of PBS; BDNF, intraportal administration of BDNF.

of islets (Fig. 3A). In addition, BDNF-like immunoreactivity was co-localised with glucagon-like immunoreactivity in islets (Fig. 3B).

Effect of central BDNF administration on glucose metabolism

Central administration of BDNF significantly decreased blood glucose at 15 min after glucose loading compared to PBS administration (n = 6 per group, $P < 0.05$; Fig. 4). There was no significant difference in plasma insulin concentrations between the two groups (Fig. 4).

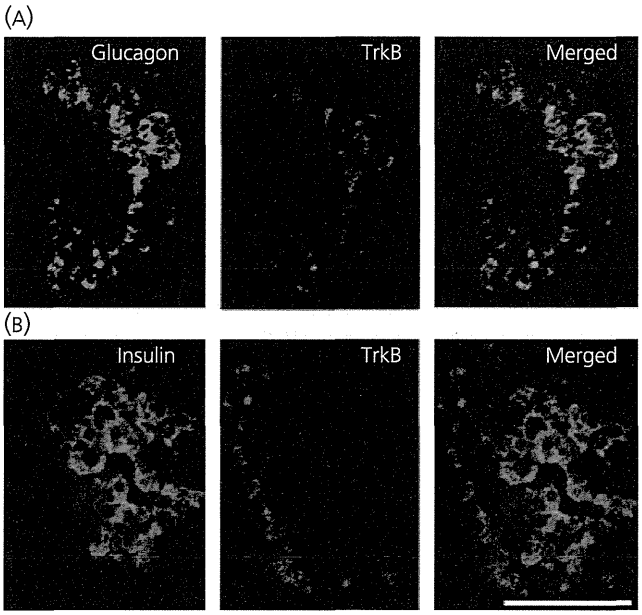

Fig. 2. TrkB are co-localised with α cells in islets. Representative glucagon (A) and insulin (B) staining (left row), tyrosine-related kinase B (TrkB) staining (middle row) and merged (right row) performed with pancreas sections derived from each group. Scale bar = 100 μm

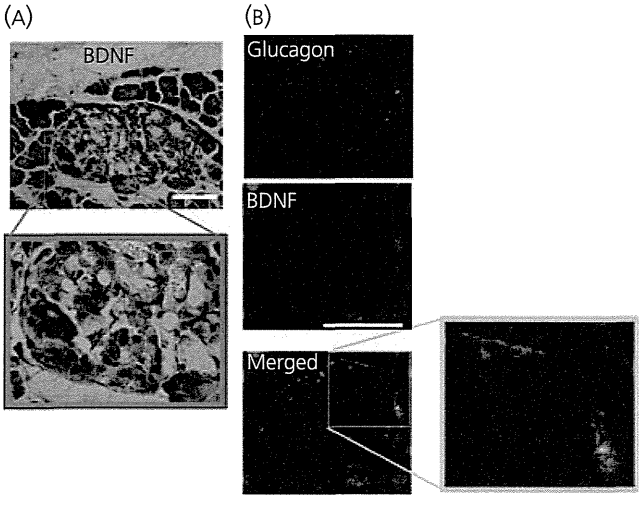

Fig. 3. Brain-derived neurotrophic factor is also co-localised with α cells in islets. (A) Representative double-staining (A) with haematoxylin and eosin and glucagon immunostaining (left row). (B) Representative immunostaining for glucagon (upper), brain-derived neurotrophic factor (BDNF) (middle) and merged (lower) in pancreas sections derived from each group. Scale bar = 100 μm.

Effect of BDNF on glucagon and insulin secretion after pancreatic efferent denervation

We investigated whether BDNF regulates glucagon or insulin secretion via the pancreatic efferent nerves because it is known that i.v. glucose infusion tends to increase activity of the VPN and reduce activity of the SPN (13). Portal glucagon was decreased by the i.c.v. administration of BDNF and this decrease was significantly suppressed by pancreatic efferent denervation (n = 6 per group, $P < 0.05$; Fig. 5A). Peripheral glucagon, portal and peripheral insulin levels were not altered by BDNF or efferent denervation (Figs 5B and 4D).

Effect of intraportal administration of GLP-1 on c-fos expression in the VMH, hypothalamic and plasma BDNF, peripheral GLP-1, and portal and peripheral glucagon levels

The intraportal administration of GLP-1 induced c-*fos* expression in the VMH compared to PBS administration (Fig. 6A). The GLP-1 treatment increased the mean number of c-*fos*-immunoreactive cells more than PBS treatment (n = 6 per group, $P < 0.05$; Fig. 6B). Intraportal administration of GLP-1 significantly increased the hypothalamic BDNF level in the hypothalamus (Fig. 6C). By contrast, no change in peripheral BDNF and GLP-1 levels was found following intraportal administration of GLP-1, indicating that this dose of GLP-1 does not affect the circulated GLP-1 level (Fig. 6D,E). Furthermore, intraportal infusion of GLP-1 decreased portal (n = 6 per group, $P < 0.05$; Fig. 6F) but not peripheral glucagon levels (Fig. 6G) compared to PBS treatment.

Effect of BDNF antibody pre-treatment on the GLP-1-induced decrease in portal glucagon level

Intraportal administration of GLP-1 significantly decreased the portal glucagon level compared to the PBS treatment (n = 6 per group, $P < 0.05$; Fig. 7A), and pre-treatment of BDNF antibody suppressed the GLP-1-induced reduction of portal glucagon level (n = 6 per group, $P < 0.05$; Fig. 7A). The central administration of BDNF antibody alone did not affect portal glucagon level compared to the control level (Fig. 7A). Moreover, there was no significant difference in peripheral glucagon, portal and peripheral insulin levels among all groups (Fig. 7B–D).

Effect of BDNF antibody pre-treatment on the GLP-1-induced increase in pancreatic BDNF level

Intraportal administration of GLP-1 increased significantly pancreatic but not plasma BDNF levels compared to PBS treatment, and pre-treatment of BDNF antibody inhibited the GLP-1-induced elevation of pancreatic BDNF level (n = 6 per group, $P < 0.05$; Fig. 8). The central administration of BDNF antibody alone did not affect

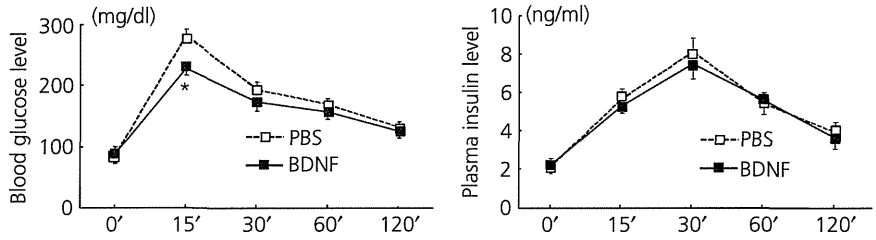

Fig. 4. Effects of i.c.v. administration of brain-derived neurotrophic factor (BDNF) on glucose metabolism. Blood glucose (left) and plasma insulin (right) levels during the glucose tolerance tests in each group (n = 6). *P < 0.05 versus phosphate-buffered saline (PBS) group. Treatment groups: PBS, i.c.v. administration of PBS; BDNF, i.c.v. administration of BDNF.

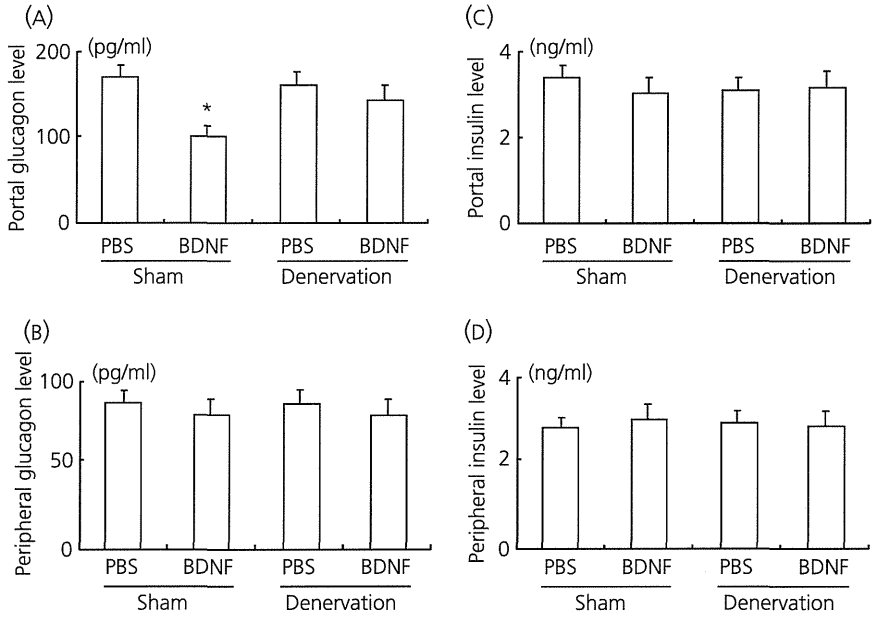

Fig. 5. Effects of i.c.v. administration of brain-derived neurotrophic factor (BDNF) on glucagon and insulin levels in sham-operated and pancreatic efferent denerved rats. Portal (A) and peripheral (B) glucagon levels in sham operation and pancreatic efferent denervation. Portal (C) and peripheral (D) insulin levels in sham operation and pancreatic efferent denervation. *P < 0.05 versus phosphate-buffered saline (PBS)-Sham group. Treatment groups: PBS-Sham, intraportally administered PBS, given sham operation; BDNF-Sham, intraportally administered BDNF, given sham operation; PBS-Denervation, intraportally administered PBS, given pancreatic efferent denervation; BDNF-Denervation, intraportally administered BDNF, given pancreatic efferent denervation.

the pancreatic BDNF level compared to the control level (Fig. 8). In addition, there was no significant difference in pancreatic and plasma BDNF levels among all groups (Fig. 8).

BDNF antibody treatment abolished the GLP-1-induced improvement in glucose metabolism

An i.p. glucose tolerance test was performed to evaluate the effects of BDNF antibody treatment on glucose metabolism. The intraportal administration of GLP-1 significantly decreased blood glucose and BDNF antibody inhibited the GLP-1-induced decrease in blood glucose at 15 and 30 min after glucose loading (n = 6 per group, P < 0.05; Fig. 8). There were no significant differences in plasma insulin concentrations between groups (Fig. 9).

Discussion

Glucagon released from the α cells, a major determinant of hepatic glucose production, has been shown to be under the control of insulin levels within the islets (14, 15). There is a general consensus that fasting hyperglycaemia in diabetes is a direct result of dysregulated hepatic glucose production. Diabetic subjects have elevated fasting glucagon levels that can be 50% greater than nondiabetic individuals, indicating that insensitivity of the α cells might be observed in diabetic patients with fasting hyperglycaemia (16, 17). Thus, there is considerable evidence for abnormal glucagon secretion in diabetes. However, it is not clear whether this is an inherent characteristic of the diabetic α cell or the result of abnormalities in extra α cell-regulatory forces. We focused on

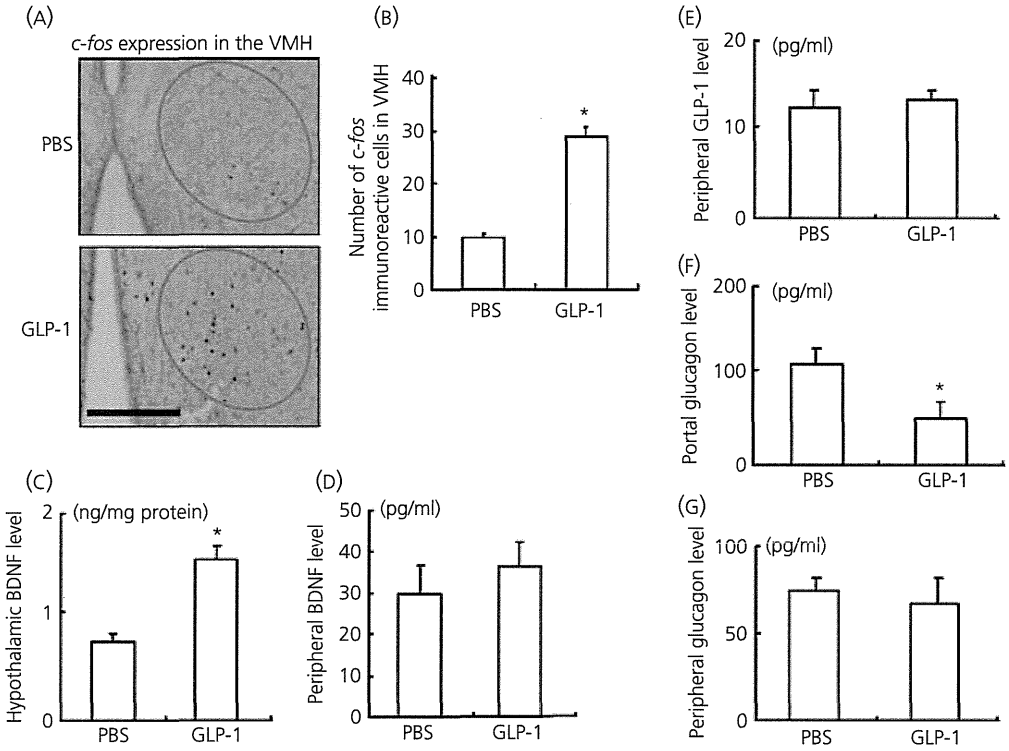

Fig. 6. Effects of intraportal administration of glucagon-like peptide (GLP)-1 on hypothalamic brain-derived neurotrophic factor (BDNF) contents and portal glucagon level. Representative immunostaining for c-*fos* (A) and comparison of the average number of c-*fos*-immunoreactive cells (B) in the ventromedial nucleus of the hypothalamus (VMH) following intraportal administration of the GLP-1 or phosphate-buffered saline (PBS). Hypothalamic (C) and peripheral (D) BDNF levels following intraportal administration of the GLP-1 or PBS. Peripheral GLP-1 level (E), portal (F) and peripheral (G) glucagon levels following intraportal administration of the GLP-1 or PBS. *P < 0.05 versus phosphate-buffered saline (PBS) group. Treatment groups: PBS, intraportal administration of PBS; GLP-1, intraportal administration of GLP-1.

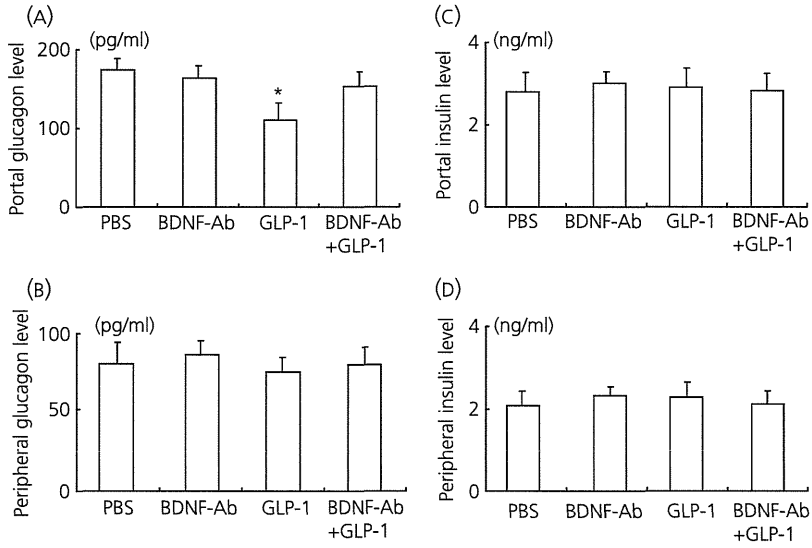

Fig. 7. Effects of i.c.v. administration of brain-derived neurotrophic factor (BDNF) antibody on glucagon-like peptide (GLP)-1-induced glucagon secretion. Portal (A) and peripheral (B) glucagon levels in each group (n = 6). Portal (C) and peripheral (D) insulin levels in each group (n = 6). *P < 0.05 versus phosphate-buffered saline (PBS) group. Treatment groups: PBS, i.c.v. administration of PBS, followed by intraportally administered PBS; BDNF-Ab, i.c.v. administration of BDNF antibody, followed by intraportally administered PBS; GLP-1, i.c.v. administration of PBS, followed by intraportally administered GLP-1; BDNF-Ab + GLP-1, i.c.v. administration of BDNF antibody, followed by intraportally administered GLP-1

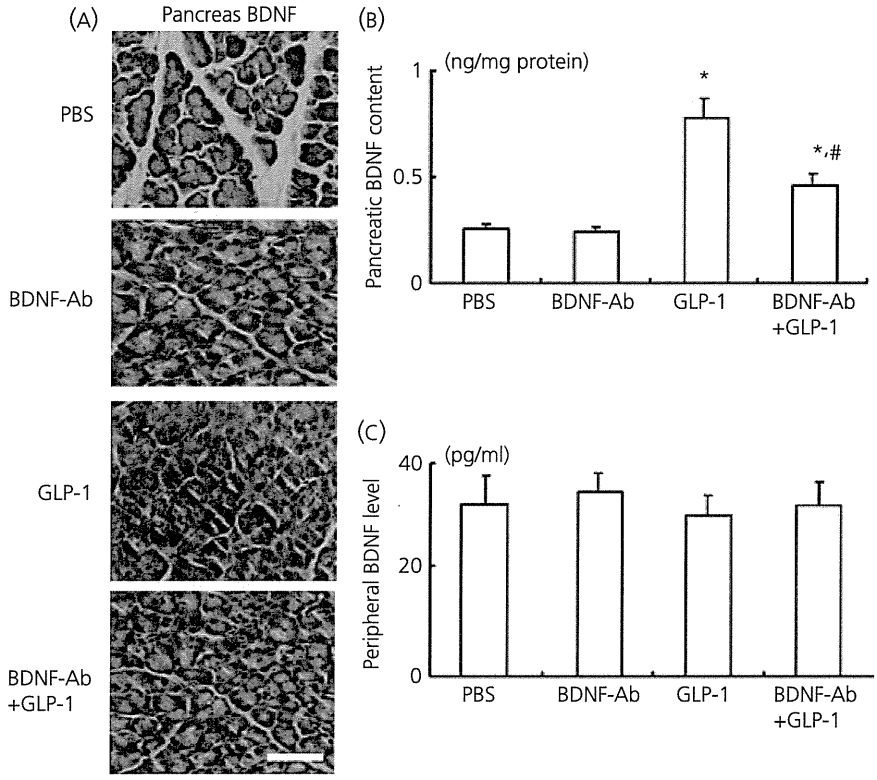

Fig. 8. Effects of i.c.v. administration of brain-derived neurotrophic factor (BDNF) antibody on glucagon-like peptide (GLP)-1-induced BDNF expression in the pancreas. (A) Representative haemotoxylin and eosin staining and immunostaining for BDNF in pancreas sections derived from each group. Scale bar = 100 μm. Pancreatic (B) and plasma (C) BDNF levels in each group (n = 6). *P < 0.05 versus phosphate-buffered saline (PBS) group, #P < 0.05 versus GLP-1 group. Treatment groups: phosphate-buffered saline (PBS), i.c.v. administration of PBS, followed by intraportally administered PBS; BDNF-Ab, i.c.v. administration of BDNF antibody, followed by intraportally administered PBS; GLP-1, i.c.v. administration of PBS, followed by intraportally administered GLP-1; BDNF-Ab + GLP-1, i.c.v. administration of BDNF antibody, followed by intraportally administered GLP-1.

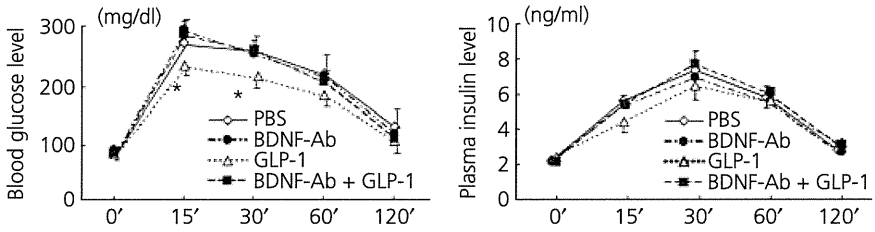

Fig. 9. Effects of i.c.v. administration of brain-derived neurotrophic factor (BDNF) antibody on glucose metabolism. Blood glucose (left) and plasma insulin (right) levels during the glucose tolerance tests in each group (n = 6). *P < 0.05 versus phosphate-buffered saline (PBS) group. Treatment groups: PBS, i.c.v. administration of PBS, followed by intraportally administered PBS; BDNF-Ab, i.c.v. administration of BDNF antibody, followed by intraportally administered PBS; glucagon-like peptide (GLP)-1, i.c.v. administration of PBS, followed by intraportally administered GLP-1; BDNF-Ab + GLP-1, i.c.v. administration of BDNF antibody, followed by intraportally administered GLP-1.

determining whether the central and peripheral nervous system actually regulates glucose metabolism.

A previous study has reported that BDNF decreases glucagon secretion in isolated mouse pancreatic islets without affecting insulin secretion (8). We found that the central administration of BDNF reduced portal glucagon but not insulin levels, suggesting that the central infusion of BDNF might improve glucose metabolism without enhancing insulin secretion from the pancreas. It has been known that BDNF is present in pancreatic nerves and overexpressed by islet cells and exocrine pancreatic cells in chronic pancreatitis (18). We observed that the central infusion of BDNF increased pancreatic BDNF content whereas it did not alter the peripheral plasma BDNF level, indicating that circulating BDNF does not affect the expression of BDNF in the pancreas. Considering that the central infusion of BDNF decreased portal glucagon levels and did not lower blood glucose levels of normal rodents as well as diabetic

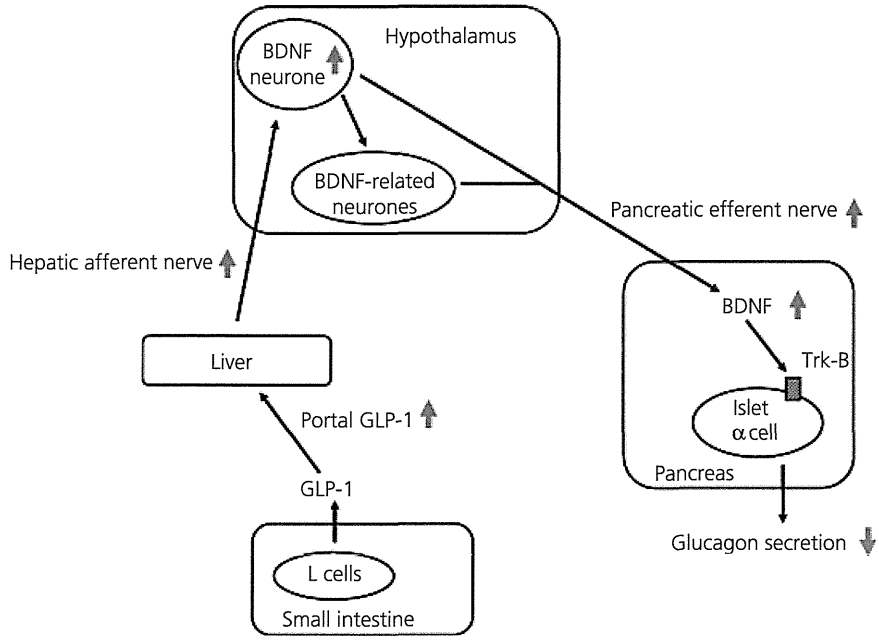

Fig. 10. Proposed model for regulation of glucagon secretion from α cells by glucagon-like peptide (GLP)-1. GLP-1 secreted from L cells in the small intestine stimulates brain-derived neurotrophic factor (BDNF) neurones in the hypothalamus via the activation of hepatic afferent nerves. Activated BDNF neurones and/or BDNF-related neurones stimulate efferent nerves to the pancreas. Furthermore, BDNF is released from the nerve terminals of these efferent nerves and suppresses glucagon secretion from α cells via tyrosine-related kinase B. 'BDNF-related neurones' indicates neurones stimulated by BDNF. Red arrow, positive direction of regulation; blue arrow, negative direction of regulation.

rodents induced by streptozotocin, it is possible that the centrally administered BDNF activates the peripheral nervous system to the pancreas and BDNF released from pancreatic peripheral nerve terminals might directly modulate the function of pancreatic α cells, in accordance with our immunohistochemical study showing that α cells were co-localised with BDNF (2, 8, 19). Additionally, we showed that TrkB, BDNF receptors were detected specifically in α cells but not β cells. This is in accordance with a previous report, in which TrkB was detected in the pancreatic α cells of rats and humans (9). However, we observed that the i.c.v. administration of BDNF decreased glucagon levels in the portal system, and not in the peripheral system, in accordance with a previous study (20). There are some reasons why changes in portal glucagon levels are not associated with changes in peripheral glucagon levels. One possible explanation is that hepatic glucagon clearance is linear up to very high levels of the hormone at portal vein levels (21). The other is that the portal vein exposes the liver to high concentrations of glucagon before it is diluted by the glucagon-poor blood of the systemic circulation. Thus, we propose that BDNF is one of the regulators of glucagon secretion.

Retrograde transneuronal viral tracing studies have provided strong anatomical evidence suggesting that hypothalamic neurones project to the pancreas (22). Two systems provide excitatory inputs to the glucagon cells of the pancreas: one is transmitted via the VPN system and the other via the SPN system to the glucagon-producing cells of the pancreas, each operating via a different receptor mechanism to elicit the release of glucagon into the blood stream (23). Considering the present data indicating that BDNF-positive fibres are present in the vicinity of α cells, within the islets, it is suggested that BDNF in the central nervous system is an important regulator of glucose metabolism in peripheral organs via 'inter-tissue communication', and the hypothalamus is an essential brain region that directs this system (24). BDNF is produced in various peripheral target tissues of the peripheral nervous system and binds to the TrkB (25). Furthermore, anterograde transport of endogenous BDNF by neurones was confirmed in adult rats, and it was shown that BDNF is transported by neurones toward peripheral targets (26). The present study demonstrates that VPN and SPN denervation to the pancreas abolishes the BDNF-induced decrease in portal glucagon levels, indicating that the excitatory input to the α cells of islets via two peripheral nervous systems from the hypothalamus might release BDNF and that this release could suppress the secretion of glucagon from the α cells. Moreover, the reduction of the pancreatic glucagon concentration is likely to contribute to the hypoglycaemic action of BDNF in obese mice because an increase in glucagon levels causes elevated hepatic glucose production (27–29). BDNF may preferentially reduce plasma glucose levels of obese diabetic mice compared to that of normal mice by its suppressive effect on glucagon secretion because an increase in the plasma glucagon/insulin ratio plays an important role in enhanced hepatic glycogenolysis, gluconeogenesis and hyperglycaemia in obese diabetic mice (30). Another study demonstrated that i.c.v. administration of BDNF in doses as small as 15 μg/mouse (approximately 300 μg/kg) proved to be effective in lowering blood glucose concentrations (31). Furthermore, i.c.v. administration of only 1.5 μg/mouse (approximately 30 μg/kg) BDNF was also found to be effective (31). Such doses are approximately 1 : 100 and 1 : 1000 of those used in s.c. administration and were not effective when

delivered peripherally (31). These data are compatible with the present results showing that BDNF acts on the brain and induces a hypoglycaemic effect via the peripheral nervous system.

There are effective antidiabetic drugs such as dipeptidyl peptide-IV (DPP-IV) inhibitors that inhibit glucagon secretion. In reality, both exogenous insulin and sulphonylureas inhibit glucagon secretion at least partially, although it is not clear how much this contributes to their treatment effect (32, 33). The glucagon family such as GLP-1 and glucose-dependent insulinotrophic polypeptide (GIP) is rapidly inactivated by the ubiquitous protease DPP-IV and the half-life of the bioactive peptide is 1–2 min (34). DPP-IV inhibitors are orally available small molecules that prolong the activity of GLP-1 and GIP. GLP-1 is mainly synthesised in the L cells of small intestine, whereas GIP is secreted from K cells in the small intestine. These hormones potentiate the acute effects of glucose on pancreatic α and β cells; thus stimulating insulin secretion and only GLP-1 inhibits glucagon secretion in a glucose-dependent manner (35, 36). Importantly, because the suppression of glucagon by GLP-1 does not occur at hypoglycaemic plasma glucose concentrations, GLP-1 does not impair the counter-regulatory defence against hypoglycaemia (37). Our data showing that glucose metabolism is not affected in the fasting condition after the portal administration of GLP-1 or the central administration of BDNF are very much in line with the absence of adverse events of hypoglycaemia in diabetic patients treated with DPP-4 inhibitors or GLP-1 receptor agonists resistant to DPP-4 (37, 38). There is still the question regardig the mechanism by which this occurs because most studies have not localised the GLP-1 receptor to α-cells (39). Thus, alternative explanations, such as indirect mediation via neural regulation, have been proposed. Our previous study demonstrated the DPP-IV inhibitor-induced rise in portal GLP-1 levels acts in the hypothalamus via the hepatic vagal afferent nerve to regulate feeding behaviour (11). We found that the intraportal infusion of GLP-1, which did not influence circulating GLP-1 and BDNF levels, increased the hypothalamic BDNF as well as *c-fos* expression in the VMH. In addition, this treatment decreased portal (but not peripheral) glucagon levels. Furthermore, the i.c.v. administration of BDNF antibody attenuated the GLP-1-induced decrease in portal glucagon levels and the increase in pancreatic BDNF content. These results suggest that the rise of portal GLP-1 levels could activate BDNF synthesis in the hypothalamus, elevate BDNF release in the pancreas, including islet areas probably via terminals of efferent pancreatic nerves from the hypothalamus, and decrease glucagon secretion from α cells (but not insulin) from β cells. The present work using the i.p. glucose tolerance test indicates the BDNF antibody suppressed GLP-1-induced improvement of glucose metabolism without affecting insulin secretion. The possibility that the brain and pancreas operate in concert to control glucagon secretion is consistent with the reported similarities found between the mechanism used by glucose-sensing β cells and their neuronal counterparts within the VMH (40). A previous study indicated that insulin within the VMH appeared to regulate glucagon secretion and blockade of insulin action in the VMH caused an immediate increase in plasma glucagon in the absence of a decline in insulin secretion. These findings support the view that hyperglucagonaemia was induced at the level of the VMH rather than the islets and raise the possibility that impaired BDNF action in the VMH may contribute to disordered systemic glucose regulation. It was observed that basal glucagon secretion was significantly increased in VMH-lesioned rats, compatible with our results showing that the inactivation of BDNF synthesised mainly in the VMH with BDNF antibody infusion suppressed the GLP-1-induced reduction of portal glucagon level (20).

In conclusion, we have shown that the increase of the portal GLP-1 level activates BDNF neurones in the hypothalamus probably via the afferent nerve from the liver, and this activation reduces glucagon secretion via efferent nerves to the pancreas, which improves glucose metabolism (Fig. 10). We need to undertake further studies to investigate: (i) which type of cells are the physiological source of BDNF affecting glucagon secretion *in vivo*; (ii) whether there are any other functions of BDNF influencing physiology or postnatal development of pancreatic α cells; (iii) what types of intracellular signalling molecules are involved in the suppressive effect of BDNF on glucagon secretion; and (iv) to what extent is BDNF useful for treatment of diabetes mellitus. These studies may help our understanding of the physiological function of BDNF on glucose metabolism and raise the possibility that an insufficiency of BDNF action is concerned in part with the aetiology or exacerbating factors of diabetes mellitus.

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グレリンによる神経求心作用

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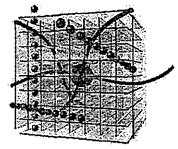

Summary

グレリンは主に胃から産生・分泌されるペプチドホルモンで，成長ホルモン分泌，食欲，胃液分泌および腸管運動の亢進，血圧降下や心拍出量増加，抗炎症など多彩な生理作用を有する．これらは，グレリン受容体(GHS-R)を介した直接作用と迷走神経を介した作用が考えられている．最近，いくつかの消化管ホルモンは迷走神経求心系を介して脳への情報を伝達することが示されており，実際，迷走神経切断ラットでは，摂食またはGH分泌に対するグレリンの作用は消失する．迷走神経求心路は，摂食やGH分泌に対するグレリンシグナルを伝達する主要な経路である．

Key words

◎グレリン　◎摂食亢進ペプチド
◎迷走神経　◎グレリン受容体

はじめに

摂食とエネルギー代謝調節は，視床下部や大脳辺縁系といった中枢神経系と，消化管，膵臓，肝臓，脂肪組織および筋を含む末梢臓器で産生される摂食亢進物質と抑制物質の複雑な相互作用により調節されている．食物が消化管内に入ると，機械的な伸展と栄養素の流入による直接的または迷走神経求心路を介したシグナルにより，摂食調節に作用するペプチドホルモンの分泌が変化する．ほとんどすべての消化管ペプチド(コレシストキニン，グルカゴン様ペプチド1：GLP-1，ペプチドYY，アミリン，オキシントモジュリン，インスリンなど)は食欲抑制の作用を持っており，末梢で産生される摂食亢進シグナル分子は胃体部で産生されるグレリンのみである．グレリンは視床下部の摂食抑制ニューロンへの抑制系シナプス形成を増加し，興奮系シナプス形成を抑制することで，摂食調節の神経回路網の可塑性にも関与している．腸管で産生される摂食抑制ペプチドのコレシストキニン，ペプチドYYとGLP-1は，迷走神経求心路の電気活動を興奮させ，延髄に情報を伝達している．本稿では，胃で産生され，摂食およびエネルギー代謝調節に機能するグレリンの

中枢への情報伝達機構を中心に，迷走神経系のエネルギー代謝調節に果たす役割について概説する．

グレリン

グレリンは，オーファンG蛋白共役型受容体のひとつであったGHS-R (growth hormone secretagogue receptor)の内因性リガンドとして，1999年に児島・寒川により，ラットの胃から分離同定された生理活性ペプチドである[1]．グレリンは28アミノ酸残基よりなるペプチドで，3番目のセリン残基の側鎖がn-オクタン酸によりアシル化されている．このn-オクタン酸の修飾が，GHS-Rを介するグレリンの活性発現に必須となっている．グレリンのアシル化修飾は血中で容易に解離し，デスアシル体となる．血中に占めるグレリンの割合は1～2割である．グレリンは，胃体部内分泌細胞(X/A-like細胞)で産生され血中に分泌される[2]．グレリンは胃に最も多く発現しており，そのほか，小腸，大腸，膵臓，視床下部，胎盤，腎臓，副腎，脂肪細胞などにも発現している[1]．

グレリンの生理作用

グレリンは，ヒトおよびラットなどのげっ歯類で*in vivo*と*in vitro*ともに強力な成長ホルモン分泌促進作用，および摂食亢進作用を示し，これらは脳室内投与だけでなく，静脈内や腹腔内投与といった末梢投与でも同様にみられる[1)3)]．また，グレリンは胃液の分泌促進，胃から大腸までの消化管の運動促進といった消化管への作用，心拍数の変化なしに起こる血圧降下や心拍出量の増加といった循環器系への作用，免疫細胞からの炎症性サイトカイン放出抑制といった抗炎症作用，骨新生作用などの多彩な生理作用が明らかになっている(**図1**)．このようなグレリンの多彩な生理作用は，血中を循環するグレリンの直接作用とも考えられるが，後で示すように，迷走神経経由で延髄に到達したグレリン情報が，一部は上位中枢へと向かい，摂食調節や高次機能の制御を，また，一部は延髄背内側核を介して末梢臓器制御を行うといったメカニズムも考えられる．

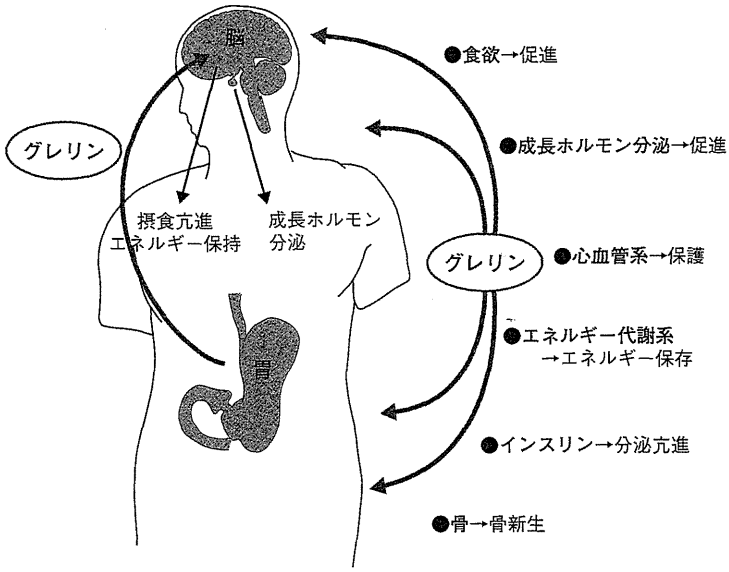

図1　グレリンの生理作用

グレリンの循環器系への作用

グレリンは内皮非依存性の血管拡張作用を示し，グレリン投与により，心後負荷の軽減，心拍出量増加が起こる．グレリンを投与すると心拍数の変動を伴わずに降圧効果がみられることや，グレリンの脳室内投与の結果から，グレリンは交感神経を抑制し，心拍数や血圧を調節することが明らかとなっている．心筋梗塞モデルラットにおいて，グレリン投与は梗塞後の心臓交感神経活動増加を抑制し，急性期不整脈による死亡を低下させた．

グレリンの受容体(GHS-R)は，心房，心室，血管などにも発現が確認されており，グレリンの循環器系への直接作用が示唆されている．グレリンは，一部，ERK1/2とPI3-kinase/Aktの活性化を介して，心筋細胞，血管内皮細胞のアポトーシスを抑制することが報告されている．

また，グレリンはGH分泌を促進し，GH/IGF-1を介して心筋収縮増大，血管拡張，心筋構築促進に作用している．慢性心不全モデルラットにグレリンを慢性投与すると，心拍出量の増加，左室駆出率の増加，左室リモデリング進展の抑制がみられ，体重および骨格筋量増加も観察された．慢性心不全患者においても，グレリン投与は代償性心肥大を促進させ，心機能・心筋構築を改善させた．また，グレリン投与によって体重は増加し，骨格筋量は有意に増加した．さらに，グレリン投与は血中のノルアドレナリンを著明に低下させた．グレリンは交感神経抑制作用を介しても心機能改善に働くと考えられる[4]．最近の報告では，グレリンは，心不全で増加するTNF-αやミオスタチンを減少させ，骨格筋萎縮を改善することが示された[5]．

グレリンの中枢への情報伝達機構

GHS-Rは，下垂体，視床下部，海馬に多く発現し，膵臓，甲状腺，脾臓，心筋，副腎，胃，小腸，大腸など広範に発現している[6][7]．GHS-Rは大多数の迷走神経求心性ニューロンで産生され，末梢に軸索輸送されている[8]．迷走神経は，内臓からの情報を中枢へ，また中枢からの情報を内臓へ伝達している．迷走神経は運動神経と感覚神経の両方の線維からなるが，約90％が無髄の求心線維であり，これらの神経終末は，消化管粘膜および粘膜下，肝門脈周囲などに分布し，消化管の物理・化学的刺激，門脈内浸透圧の一部だけでなく，消化管ホルモンの中枢への情報伝達にも機能している[8)-11)]．

迷走神経遮断後にグレリンを末梢投与すると，グレリンによる摂食亢進作用や交感神経抑制は起こらず，グレリン投与により誘導される視床下部弓状神経核の活性化もみられない．また，グレリンをラット静脈内に投与すると，迷走神経胃枝求心線維の発火頻度が抑制される(**図2**)．つまり，胃で産生・分泌されたグレリンが迷走神経に直接作用することにより，情報を中枢へ伝達している．

末梢グレリンの中枢内での情報伝達機構

末梢グレリン情報は，迷走神経求心神経により延髄孤束核へ入力した後，何らかの物質を介して上位中枢へと伝達される．延髄から視床下部への神経路を遮断した両側中脳切断ラットでは，末梢投与したグレリンの摂食亢進作用が消失することから，グレリンの情報伝達には，延髄から視床下部に至る神経路が重要であると考えられる[9]．延髄孤束核ではさまざまな神経伝達物質の存在が知られているが，なかでもノルアドレナリンは低血糖時に反応して摂食行動を制御することや，視床下部弓状核の神経細胞とシナプス結合していることが知られている．グレリンを末梢投与したラットの延髄孤束核では，ドパミンをノルアドレナリンに変換するドパミンβヒドロキシラーゼ(DBH)のmRNA発現が有意に増加する．さらに，グレリン末梢投与後の視床下部弓状核では，ノルアドレナリン濃度が一過性に上昇する(**図2**)．また，ノルアドレナリン受容体α_1あるいはβ_2に対する遮断薬をラット脳室

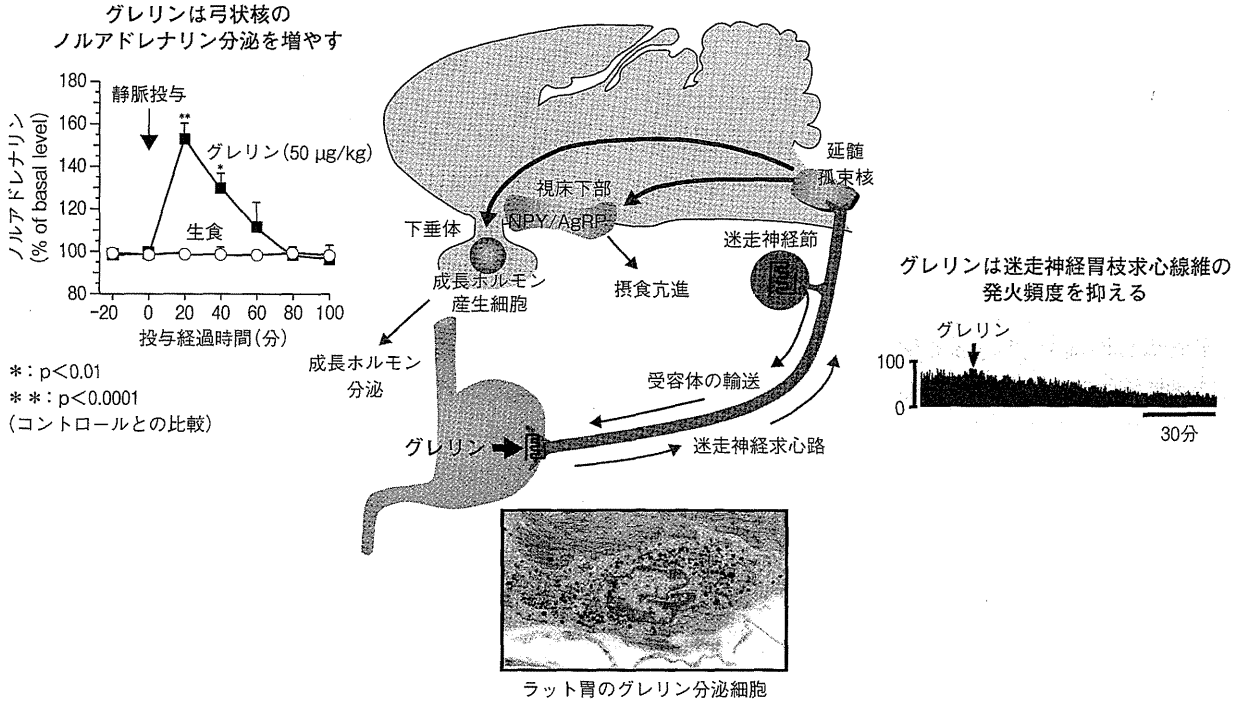

図2　グレリンの迷走神経求心路

内に投与した後にグレリンを末梢投与すると，グレリンによる摂食亢進作用が減衰する．これらの結果から，末梢投与したグレリンは延髄孤束核でのノルアドレナリン産生および視床下部弓状核でのノルアドレナリン分泌を増加させ，さらに分泌されたノルアドレナリンがα_1およびβ_2受容体を介して摂食行動を引き起こしている可能性が示唆される．

これらの結果より，末梢投与されたグレリンは，迷走神経末端のグレリン受容体と結合し，迷走神経の電気活動を抑制することで，情報を延髄孤束核に伝達する．同時に延髄孤束核のノルアドレナリンニューロンを刺激して，視床下部弓状核で分泌されるノルアドレナリン，また，ノルアドレナリン受容体を介して活性化されたニューロペプチドY(NPY)やアグーチ関連蛋白質(agouti-related protein：AgRP)ニューロンを介して，最終的に末梢グレリンの摂食亢進に関与している可能性があることが示唆される(**図2**)．

おわりに

摂食行動は，末梢の液性因子，自律神経調節因子および学習，記憶，情動などの因子が中枢で統合されることにより複雑かつ精巧に制御されている．われわれは，摂食亢進ペプチド；グレリンの研究を通じて，迷走神経求心路を情報伝達経路とする末梢と中枢の機能連関とその分子機構について研究してきた．迷走神経系は末梢のエネルギー代謝状況を脳に伝える重要なメディエーターであり，神経系の機能不全が肥満やレプチン抵抗性の一因となっている可能性も考えられる．迷走神経とエネルギー代謝異常の病態との関連を明らかにしていくことにより，メタボリックシンドロームや生活習慣病の予防や治療へとつながる可能性が期待される．

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COMMON VARIANTS IN *BCL9* GENE AND SCHIZOPHRENIA IN A JAPANESE POPULATION: ASSOCIATION STUDY, META-ANALYSIS AND COGNITIVE FUNCTION ANALYSIS

UOBIČAJENE VARIJANTE *BCL9* GENA I ŠIZOFRENIJA U JAPANSKOJ POPULACIJI: STUDIJA POVEZANOSTI, METAANALIZA I ANALIZA KOGNITIVNIH FUNKCIJA

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Summary

Background: Schizophrenia is a relatively common disorder, with a lifetime prevalence of about 1%. Family history is the most important risk factor for schizophrenia, consistent with a genetic contribution to its etiology. Recent human genetic studies reported that some common variants located within *BCL9* are associated with schizophrenia in the Chinese population, but not associated with bipolar disorder in the Caucasian population.
Methods: Single nucleotide variant (SNP) prioritization sample was comprised of 575 patients with schizophrenia and 564 healthy controls with no personal or family history of psychiatric illness. For SNP association analysis, we used an independent Japanese sample set (replication sample) comprising 1464 cases and 1171 controls. For the analysis of cognitive performance, we investigated 115 cases and 87 controls using Continuous Performance Test (CPT-IP) and the Wisconsin Card Sorting Test Keio version (WCST). Meta-

Kratak sadržaj

Uvod: Šizofrenija je relativno čest poremećaj, sa rasprostranjenošću od oko 1% u ukupnoj populaciji. Porodična istorija bolesti predstavlja najvažniji faktor rizika za nastanak šizofrenije, što je u skladu sa genetičkom osnovom njene etiologije. Nedavne genetičke studije pokazuju da su neke uobičajene varijante u okviru gena *BCL9* u vezi sa šizofrenijom u kineskoj populaciji, ali ne i sa bipolarnim poremećajem u populaciji belaca.
Metode: Uzorci za analizu tačkastih polimorfizama (SNP) potiču od 575 pacijenata sa šizofrenijom i 564 zdravih kontrolnih subjekata bez lične ili porodične istorije psihijatrijskih oboljenja. Za SNP analizu korišćen je nezavisni japanski set uzorak (replikacioni uzorak) koji sadrži 1464 slučaja bolesti i 1171 kontrolu. Za analizu kognitivnih funkcija, ispitivali smo 115 slučajeva bolesti i 87 kontrolnih slučajeva, korišćenjem kontinualnog testa funkcija (CPT-IP) i *Wisconsin Card Sorting* testa, Keio verzije (WCST). Metaanaliza je

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analysis was performed using a combined Japanese total sample (N=3735) and a Chinese sample from a previous study.
Results: In the replication sample set, we did not detect any association in 2 SNPs (rs672607 and rs10494252) and schizophrenia. Meta-analysis of rs672607 showed significant association (p-value 0.012, odds ratio 0.855). There was a significant ($p<0.01$) difference between the A/A and G carrier group of rs672607 in CPT mean d' ($p=0.0092$).
Conclusions: We were able to detect evidence for an association between rs672607 in BCL9 and schizophrenia in the meta-analysis of Japanese and Chinese populations. Additionally, this common variant may affect cognitive performance, as measured by the CPT-IP in schizophrenia patients.

Keywords: *BCL9*, Chinese, cognitive impairment, genome-wide association study, Japanese, meta-analysis, schizophrenia

urađena korišćenjem kombinovanog japanskog ukupnog uzorka (N=3735) i kineskog uzorka iz prethodne studije.
Rezultati: U replikacionom uzorku nije otkrivena nikakva veza između 2 SNP-a (rs672607 i rs10494252) i šizofrenije. Metaanaliza rs672607 je pokazala njegovu značajnu povezanost sa šizofrenijom (p-vrednost 0,012, 0,855 *odds ratio*). Utvrđena je značajna ($p<0,01$) razlika između A/A i G grupe nosilaca rs672607 u CPT srednjoj vrednosti d' ($p=0,0092$).
Zaključak: Dokazana je veza između rs672607 u genu BCL9 i šizofrenije u metaanalizi japanske i kineske populacije. Pored toga, ova zajednička varijanta može da utiče na kognitivne funkcije, što je utvrđeno testom CPT-IP kod šizofrenih bolesnika.

Ključne reči: *BCL9*, Kinezi, kognitivne funkcije, GWAS, Japanci, metaanaliza, šizofrenija

Introduction

Schizophrenia is a chronic, more or less enervating illness characterized by impairments in cognition, affect and behavior, all of which have a pronounced bizarre aspect (1). Delusions, which are generally bizarre, and hallucinations, generally auditory in type, typically occur during the clinical course of schizophrenia (2).

Schizophrenia is a relatively common disorder, with a lifetime prevalence of about 1% (3). Although the overall sex ratio is almost unbiased, males tend to have an earlier onset than females, a finding accounted for by the later age of onset in those females who lack a family history of the disease (4). Family history is the most important risk factor for schizophrenia, consistent with a genetic contribution to its etiology (5) and the heritability of schizophrenia is estimated to be 64% (6). Although genes relevant for schizophrenia or variants that may modulate risk for the disease have been identified using both linkage- and candidate-based or whole genome association studies, the genetic basis of schizophrenia is still unclear (7–10).

Recent human genetic studies reported that some B-cell CLL/lymphoma 9 gene (*BCL9*) variants are associated with schizophrenia in the Chinese population (11), but not associated with bipolar disorder in the Caucasian population (12). In addition, another study showed evidence for genetic association between common variants within *BCL9* and negative symptoms in schizophrenia patients (13). *BCL9* maps to chromosome 1q21.1 (NCBI37: 145,479,806-145,564,639), a region that was shown to be associated with schizophrenia (14). In addition, about 75% of all children with a 1q21.1 microdeletion have delayed development, particularly affecting the development of motor skills such as sitting, standing, and walking, while the intellectual disability and learning problems associated with this genetic change are usually mild (15). Furthermore, schizophrenia is significantly more common in combination with the 1q21.1 deletion syndrome, while autism is significantly more common with the 1q21.1 duplication syndrome (16).

From a biological point of view, the *BCL9* is required for efficient T-cell factor-mediated transcription in the Wnt signaling pathway (17). The Wnt signaling pathway influences neuroplasticity, cell survival, and adult neurogenesis (11), and several studies have suggested that mental disorders may involve impairments in these functions (18). As *BCL9* is indeed an attractive candidate gene for schizophrenia that has not been investigated in the Japanese population, we examined the relationship of common SNPs in *BCL9* and the risk for schizophrenia in a large Japanese case-control sample and conducted a meta-analysis between the Chinese (11) and Japanese sample set used in the current study. We also explored potential relationships between SNPs in *BCL9* and the aspects of human cognitive function.

Materials and Methods

Participants

This study was approved by the Ethics Committees of the Nagoya University Graduate School of Medicine and associated institutes and hospitals. Written informed consent was obtained from all participants. In addition, the patients' capacity to consent was confirmed by a family member when needed. Subjects with legal measure of reduced capacity were excluded. Patients were included in the study if they (1) met the DSM-IV criteria for schizophrenia, (2) were physically healthy and (3) had no mood disorders, substance abuse, neurodevelopmental disorders, epilepsy or known mental retardation. A general characterization and psychiatric assessment of subjects is available elsewhere (19). Controls were select-

ed from the general population. Control subjects had no history of mental disorders, based on questionnaire responses from the subjects themselves during the sample inclusion step, and based on an unstructured diagnostic interview done by an experienced psychiatrist during the blood collection step.

The JGWAS sample was comprised of 575 patients with schizophrenia (43.5±14.8 years (mean±s.d.), male 50%) and 564 healthy controls with no personal or family history of psychiatric illness (44.0±14.4 years (mean±s.d.), male 49.8%). All subjects were unrelated, living in the central area of the Honshu island of Japan and self-identified as members of the Japanese population.

For SNP association analysis, we used an independent Japanese sample set (replication sample) comprising 1464 cases (aged 45.9±14.2 years, male 54.5%) and 1171 controls (aged 48.06±14.48 years, male 47.3%). For the analysis of cognitive performance, we investigated 115 cases (aged 45.3±14.2 years, male 64.3%) and 87 controls (aged 26.3±7.7 years, male 63.2%).

SNP prioritization step

From the previous genetic study of *BCL9* in a Chinese population, we selected a SNP with the lowest p-value (rs672607 A>G, $p=1.23*10^{-11}$). From the JGWAS data set, there were 3 SNPs (rs17160256, rs17160264 and rs10494252) with $p<0.05$ in BCL9 and +10% region. We selected only one SNP (rs10494252 A>G, p=0.0369) from the 3 SNPs because of high r^2 (>0.95) in the Japanese population (SNPinfo Web Server, http://snpinfo.niehs.nih.gov/snpinfo/index.html).

Genotyping and data analysis

DNA was extracted from peripheral blood according to a standard protocol (20, 21). Genotyping was performed using a fluorescence-based allelic discrimination assay (Taqman, Applied Biosystems, Foster City, CA). To exclude low-quality DNA samples or genotyping probes, data sets were filtered on the basis of SNP genotype call rate (more than 90%) or deviation from the HWE in the control sample. Subjects whose percentage of missing genotypes was >10% or who had evidence of possible DNA contamination were excluded from subsequent analyses. All allele-wise association analyses (JGWAS or replication sample set) were carried out by calculating the p-values for each candidate SNP. Significance was determined at the 0.05 level using Fisher's exact test. All p-values were two-sided. In this joint analysis, p-values were generated by Cochran-Mantel-Haenszel stratified analysis, and the Breslow-Day test was performed for evaluation of heterogeneous associations as implemented in PLINK v1.07 (22). Statistical significance was set at a nominal level ($p<0.05$) in an association study. Comprehensive Meta-Analysis Version 2 Professional version (Biostat, Inc., http://www.meta-analysis.com/index.html) was used to conduct a meta-analysis of the Japanese and Chinese sample sets in rs672607.

Neurocognitive assessment

We used the Continuous Performance Test–Identical Pairs Version Release 4.0 (CPT-IP) (New CPT.exe, Copyright 1982–2004 by Barbara A. Cornblatt, All Rights Reserved). The size of the PC monitor used for the test was 10.4 inches as each letter was at least 2.2×1.5 cm (23, 24). Stimuli were flashed on the screen at a constant rate of 1 per second, with a stimulus »on« time of 50 ms. Stimuli were four-digit numbers and were presented 150 times. In each 150-trial condition, 30 of the trials (20%) were target trials and required a response. Target trials were those on which the second of a pair of two identical stimuli appeared (23). The outcome measure was a mean d'.

The Wisconsin Card Sorting Test (WCST) (25) mainly assesses executive function including cognitive flexibility in response to feedback. We used a modified and computerized version of the test: Wisconsin Card Sorting Test (Keio Version) (KWCST) (26–28). The outcome measures were numbers of categories achieved (CA), total errors (TE), and perseverative errors of Milner (PEM) and Nelson types (PEN) in the first trial. We selected outcomes in the WCST, following a prior study, which used KWCST as a measure of cognitive function (29): (1) CA, which is the number of categories for which six consecutive correct responses are achieved (eight is the maximum number of categories which can be achieved), and which is the sum measure of the level of conceptual shifts in the KWCST; (2) PEN, which is the number of incorrect responses in the same category as the immediately preceding incorrect response (maximum of 47 perseverative errors); (3) PEM, which is the number of incorrect responses in the same category as the immediately preceding correct response after the category changes; and (4) TE, which is the total number of incorrect responses.

Chlorpromazine (CPZ) equivalent doses were calculated based on the report by Inagaki et al. (30, 31). The Positive and Negative Symptom Scale (PANSS) was used to evaluate patients (32). From the sample used in the current study, we made a subset of randomly selected participants older than 18 years of age for an analysis of cognitive performance. Cognitive data analysis was done for the participants who completed both WCST and CPT-IP. We checked the effect of two SNPs (rs672607 and rs10494252) on cognitive performance measured by the CPT-IP and the WCST (115 schizophrenic patients, 87