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Protective effect of brain-derived neurotrophic factor on pancreatic islets in obese diabetic mice

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Abstract

We have previously demonstrated that brain-derived neurotrophic factor (BDNF) ameliorates glucose metabolism and energy expenditure in obese diabetic *db/db* mice. In the present study, the effect of BDNF treatment on pancreatic islets of *db/db* mice was examined, using vehicle-treated pair-fed *db/db* mice as controls. Brain-derived neurotrophic factor (10 mg/kg) or vehicle was subcutaneously administered to male *db/db* mice for 4 weeks. The food intake of vehicle-treated *db/db* mice was restricted and precisely synchronized with that of BDNF-treated *db/db* mice using a pellet pair-feeding apparatus because BDNF decreases food intake in hyperphagic mice. Repetitive administration of BDNF significantly lowered the blood glucose concentration compared with pair-fed vehicle-treated *db/db* mice. The pancreatic insulin and glucagon concentrations were measured in *db/db* mice to evaluate the effect of BDNF on the pancreas. Although the insulin concentration in the pancreas of pair-fed vehicle-treated *db/db* mice was lower than in nondiabetic control *+m/+m* mice, it was higher in BDNF-treated *db/db* mice than in vehicle-treated pair-fed *db/db* mice and comparable to the concentration in *+m/+m* mice. The glucagon concentration in the pancreas of vehicle-treated pair-fed *db/db* mice was higher than in *+m/+m* mice, and BDNF partially decreased the glucagon concentration in the pancreas of *db/db* mice compared with vehicle. Histologic analyses of pancreatic sections were performed to characterize the mechanism through which BDNF modulates the hormonal concentration in the pancreas of *db/db* mice. Although there were no significant differences in the number and total area of islets between the BDNF- and vehicle-treated groups, immunostaining with an anti-insulin antibody indicated that the islet beta-cell area in BDNF-treated *db/db* mice was larger than that in vehicle-treated pair-fed *db/db* mice. Furthermore, immunostaining with an antiglucagon antibody indicated that BDNF normalized the delocalization of non-beta cells in islets of *db/db* mice. Electron microscopic images of beta cells indicated a decrease in secretory granules in vehicle-treated pair-fed *db/db* mice; this change was reversed in BDNF-treated *db/db* mice and reached a level comparable to that found in *+m/+m* mice. These findings suggest that BDNF prevents exhaustion of the pancreas in diabetic mice by maintaining the histologic cellular organization of beta cells and non-beta cells in pancreatic islets and restoring the level of insulin-secreting granules in beta cells.

1. Introduction

Neurotrophins (NTs) are a family of secreted proteins that regulate neurite outgrowth and differentiation of certain populations of neurons in neurogenesis, in addition to modulating survival and synthesis of neurotransmitters in mature neurons [1,2]. The NTs include nerve growth factor, ciliary neurotrophic factor, brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5 [3-6]. In addition to the role of NTs in neuronal development and neurotrophic action on mature neurons, we have found that BDNF reduces food intake and lowers the blood glucose concentration in obese diabetic animal models such as *db/db* mice [7-9] through a hypoglycemic action that is independent of appetite alteration [10].

The major pathogenic features of type 2 diabetes mellitus are insulin resistance and insufficient insulin secretion [11]. In early stages of this disease, insulin secretion from the pancreas increases to compensate for insulin resistance in

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peripheral tissues such as liver and skeletal muscle [11-13]. This continuous load on the pancreas often results in progressive deterioration of beta-cell function, thereby causing type 2 diabetic patients to be dependent on insulin treatment [14]. Therefore, prevention of pancreatic dysfunction in type 2 diabetic patients is a desirable profile for an antidiabetic drug.

The pancreatic insulin concentration in BDNF-treated *db/db* mice is known to be higher than that in ad libitum–fed vehicle-treated *db/db* mice [10], but the manner in which BDNF affects pancreatic function and structure in *db/db* mice compared with precisely pair-fed control animals has not been fully characterized. In the present study, the effects of BDNF on the pancreas in *db/db* mice were evaluated, using pair-fed mice as controls, and the mechanism through which BDNF modulates the function of pancreatic islets was examined. First, the effects of BDNF on the pancreatic insulin and glucagon concentrations in *db/db* mice were examined, and subsequently the effects of BDNF on the number and total area of islets in the pancreas in *db/db* mice were evaluated. The area and distribution of beta cells and non-beta cells in *db/db* mice were determined using immunohistochemical analysis, and potential BDNF amelioration of beta-cell disorders in *db/db* mice was evaluated by electron microscopy.

2. Methods

2.1. Animals

Male C57BL/KsJ-*db/db* mice were obtained from Clea Japan (Tokyo, Japan). Treatment with BDNF or vehicle was started at 8 weeks of age (n = 8). Age-matched male C57BL/KsJ-+*m*/+*m* mice (n = 8) were used as nondiabetic controls. Animals were housed in individual cages, with a daily cycle of 12 hours each of light and darkness. Food (CE-2, Clea Japan, Tokyo, Japan) and water were given ad libitum except during the pair-feeding period. All animal experiments were conducted according to the guidelines of the Dainippon Sumitomo Pharma Committee on Animal Research.

2.2. Brain-derived neurotrophic factor treatment

Human recombinant BDNF (N-terminal methionine-free, Regeneron Pharmaceuticals, Tarrytown, NY) was administered subcutaneously. Tween 80 (0.01%) and mannitol (1%) in phosphate-buffered saline (10 mmol/L phosphate, 150 mmol/L NaCl, pH 7.0) was used as vehicle. We have previously reported that BDNF dose-dependently reduces blood glucose concentration in *db/db* mice [9]. In that study, once-daily administration of 10 mg/kg or more of BDNF significantly reduced the blood glucose concentration in *db/db* mice. Therefore, we chose 10 mg/kg as the dose of BDNF in the present study.

2.3. Synchronized pair-feeding apparatus

A synchronized pellet pair-feeding apparatus was used (Osaka Micro Systems, Osaka, Japan) to carry out experiments under precise pair-feeding conditions. It is composed of a controller, counter printer, and 8 pairs (master and slave) of cage units. There is a pellet feeder and pellet detector in each cage unit; each of the 8 cage unit pairs is independently controlled. The supply of pellets to the master cage is not limited, but the supply of pellets to the slave cage is limited to the number of pellets consumed by the mouse in the master cage. The number of pellets consumed in each cage unit is recorded per unit time. The details of this system have been described previously [10].

2.4. Measurement of blood glucose, insulin, and glucagon

Brain-derived neurotrophic factor (10 mg/kg per day, n = 8) or vehicle (n = 8) was administered subcutaneously to *db/db* mice for 4 weeks. Blood samples were collected from the tail vein, and blood glucose concentrations were measured using a Wako Glucose CII-Test (mutarotase–glucose oxidase method, Wako Chemical, Tokyo, Japan). Pancreatic tissues were weighed, homogenized, and extracted with acid ethanol solution (concentrated HCl/ethanol/distilled H_2O = 1.5:75:23.5), and then the insulin and glucagon concentrations in supernatant were measured. The plasma and pancreatic insulin concentrations were measured using an enzyme-linked immunosorbent assay (Levis-insulin-mouse, Shibayagi, Shibukawa, Japan), and the pancreatic glucagon concentration was measured using an EIA (YK090 Glucagon EIA kit, Yanaihara Institute, Shizuoka, Japan).

2.5. Histochemical staining of pancreatic tissue

Pancreatic tissues excised from *db/db* mice after BDNF or vehicle treatment for 4 weeks were fixed in 10% formalin (phosphate-buffered) and then embedded in paraffin. The tissues were sliced into 3-μm sections, and approximately 20 sections were subjected to hematoxylin-eosin (HE) staining or immunostaining analysis. All microscopic images were obtained at a magnification of $\times$100. The number and total area of islets in HE-stained sections were measured using an Image Processor Analytical Pathology (IPAP) image analyzing system (Sumisho Computer Systems, Tokyo, Japan) [15]. A binary digitized image of the islet was obtained automatically using the programmed segmentation procedure, and the islet area was quantified by tracing the border between islets and other components in the pancreas. Other sections of the pancreas were immunostained with anti-insulin, antiglucagon, or antisomatostatin antibody (Dako Japan, Kyoto, Japan). Islets with long diameters greater than 100 μm and less than 600 μm were randomly selected from each pancreas, and then the beta-cell and non–beta-cell areas of each islet (% of the total islet area) were measured using the IPAP image analyzing system. The ratio of the beta-cell area to the non–beta-cell area was calculated from these data.

2.6. Electron microscopy analysis

For electron microscopy analysis, pancreatic samples excised from *db/db* mice after BDNF or vehicle treatment

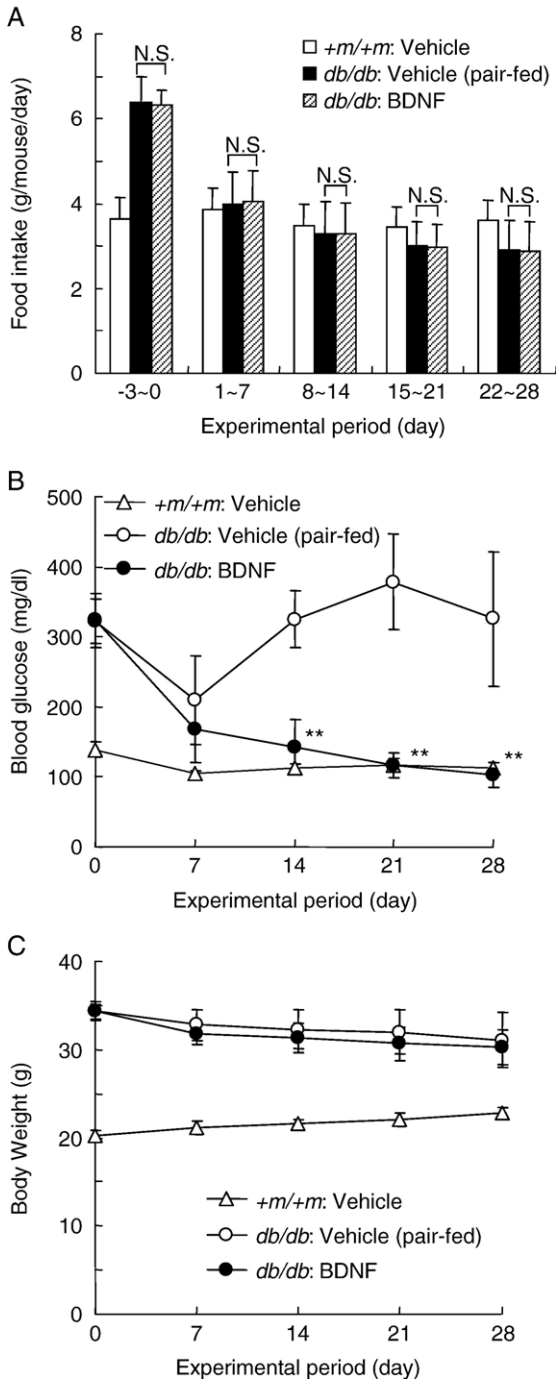

Fig. 1. Effect of BDNF on food intake (A), blood glucose (B), and body weight (C) in *db/db* mice. Brain-derived neurotrophic factor (10 mg/kg) or vehicle was administered daily to *db/db* mice housed in pellet pair-feeding apparatus for 4 weeks. Data are expressed as mean ± SD (n = 8). $^{**}P < .01$ vs vehicle-treated pair-fed *db/db* mice by analysis of variance followed by Fisher paired least squares difference. NS indicates not significant.

for 4 weeks were fixed with 2.5% glutaraldehyde and 2% osmic acid, and then dehydrated and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead acetate, and each section was observed with a JEM1200EX2 electron microscope (Japan Electron, Tokyo, Japan).

2.7. Statistical analysis

All data are presented as means ± SD. Differences between individual groups were tested using analysis of variance with Fisher paired least squares difference as a

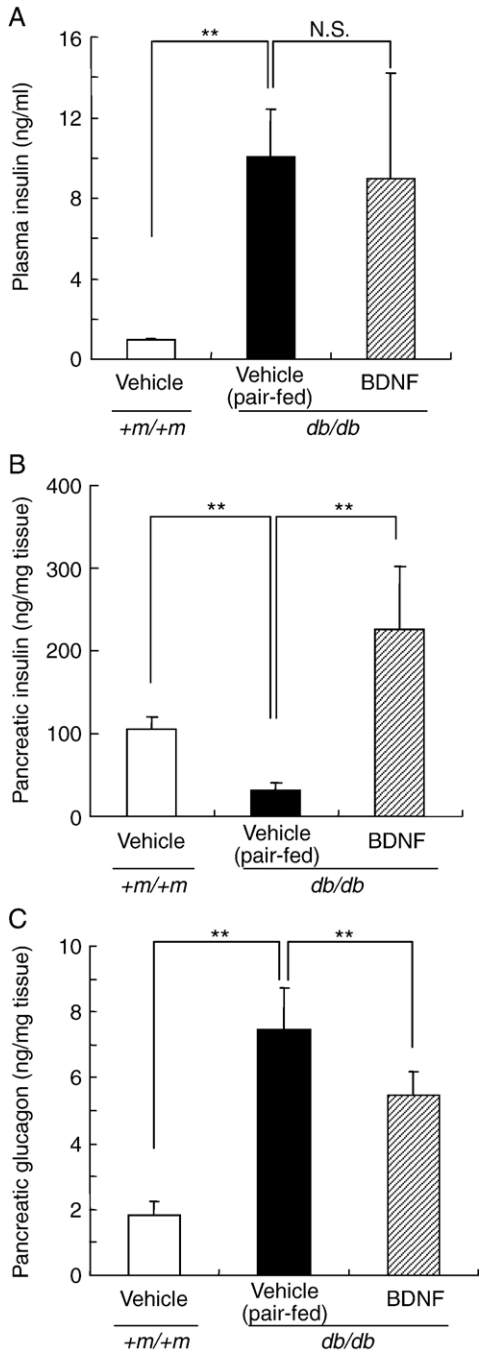

Fig. 2. Effect of BDNF on pancreas of *db/db* mice. Brain-derived neurotrophic factor (10 mg/kg) or vehicle was administered daily to *db/db* mice for 4 weeks. Plasma insulin (A) concentration was measured on day 28. At the end of the treatment, pancreas tissues were excised and extracted in acid-ethanol solution, then pancreatic insulin (B) and glucagon (C) concentration were measured. Data are expressed as mean ± SD (n = 8). $^{**}P < .01$ vs vehicle-treated pair-fed *db/db* mice by analysis of variance followed by Fisher paired least squares difference. NS indicates not significant.

Table 1
Effect of BDNF on pancreatic islets in *db/db* mice

	Islet number (count)	Total islet area (mm^2)
+m/+m: vehicle	301.4 ± 37.2	5.36 ± 0.61
db/db: vehicle (pair-fed)	613.4 ± 111.3**	13.39 ± 1.64**
db/db: BDNF	700.0 ± 164.8**	15.09 ± 3.47**

Data are expressed as mean ± SD (n = 5). Brain-derived neurotrophic factor (10 mg/kg) or vehicle was administered daily to *db/db* mice for 4 weeks. At the end of treatment, whole pancreas was resected and fixed in 10% formalin (phosphate-buffered). Total number and area of pancreatic islets in HE-stained sections were quantitatively measured using IPAP image analyzing system.

** $P < .01$ vs vehicle-treated *+m/+m* control mice by analysis of variance followed by Fisher paired least squares difference. There was no significant difference between BDNF-treated and vehicle-treated pair-fed *db/db* mice.

post hoc test. Differences were considered significant with $P < .05$.

3. Results

3.1. Effect of BDNF on blood glucose and plasma insulin concentrations in db/db mice

Brain-derived neurotrophic factor (10 mg/kg) was subcutaneously administered to obese diabetic *db/db* mice for 4 weeks, and the blood glucose and plasma insulin concentrations were measured during this period. Because BDNF has been shown to reduce food intake in hyperphagic animals in previous studies, a pellet pair-feeding apparatus was used to precisely synchronize feeding between BDNF-treated and vehicle-treated mice. As shown in Fig. 1A, good synchronization of food intake was achieved for these mice. Under the pair-feeding conditions, the blood glucose concentration in BDNF-treated *db/db* mice remained lower than in vehicle-treated pair-fed mice, and the difference between the 2 groups was found to be significant 14 days after the start of BDNF administration (Fig. 1B). There was no significant difference in body weight between BDNF- and vehicle-treated pair-fed *db/db* mice (Fig. 1C). Although the plasma insulin concentration in the vehicle-treated pair-fed *db/db* mice was higher than in nondiabetic control *+m/+m* mice, no significant difference in plasma insulin concentration was found between BDNF- and vehicle-treated pair-fed *db/db* mice after the 4-week treatment period (Fig. 2A).

3.2. Effects of BDNF administration on insulin and glucagon concentrations in the pancreas of db/db mice

Pancreatic hormonal concentrations were measured after administration of BDNF or vehicle for 4 weeks to evaluate the effect of BDNF on the pancreas. The pancreatic insulin concentration in vehicle-treated pair-fed *db/db* mice was lower than in *+m/+m* control mice (Fig. 2B), and BDNF treatment significantly increased the pancreatic insulin concentration compared with vehicle (Fig. 2B). The pancreatic glucagon concentration in vehicle-treated pair-fed *db/db* mice was higher than in normal control *+m/+m* mice, and BDNF partially but significantly suppressed the increase in pancreatic glucagon concentration in pair-fed *db/db* mice (Fig. 2C).

3.3. Effects of BDNF administration on morphological changes in pancreatic sections of db/db mice

Histologic analyses of pancreatic sections were performed to clarify the mechanism through which BDNF

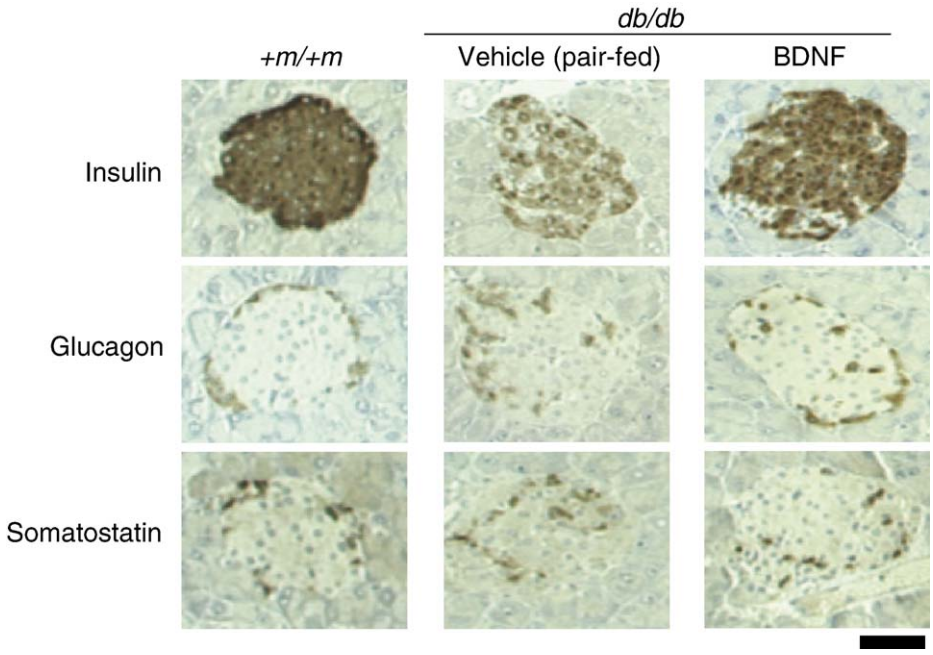

Fig. 3. Histologic analysis of pancreatic islets from age-matched vehicle-treated *+m/+m* mice (A), vehicle-treated pair-fed *db/db* mice (B), or BDNF-treated *db/db* mice (C). Brain-derived neurotrophic factor (10 mg/kg) or vehicle was administered daily to *db/db* mice for 4 weeks. At the end of treatment, pancreatic tissues were excised and fixed in 10% formalin (phosphate-buffered). Anti-insulin (upper panels), antiglucagon (middle panels), or antisomatostatin (bottom panels) immunostaining was performed (bar = 50 μm).

Table 2
Effect of BDNF on pancreatic beta cells in *db/db* mice

	Beta-cell area (%)	Non–beta-cell area (%)	Beta-cell/non–beta-cell ratio
+m/+m: vehicle	62.2 ± 3.3	7.5 ± 0.6	8.4 ± 0.9
db/db: vehicle (pair-fed)	25.5 ± 7.8††	8.8 ± 2.4	3.0 ± 0.7††
db/db: BDNF	54.7 ± 5.2†,**	5.3 ± 0.9*	9.1 ± 1.5*

Data are expressed as mean ± SD (n = 5). Brain-derived neurotrophic factor (10 mg/kg) or vehicle was administered daily to *db/db* mice for 4 weeks. At the end of treatment, whole pancreas was resected and fixed in 10% formalin (phosphate-buffered). Beta cells were identified by immunostaining analysis using anti-insulin antibody. Non-beta cells were identified by immunostaining analysis using mixed (antiglucagon, anti-somatostatin, and antipancreatic polypeptide) antibodies. Islets with a long diameter greater than 100 μm and less than 600 μm were selected randomly from each pancreas, then beta-cell area and non–beta-cell area of each islet (% of islet area) were measured using IPAP image analyzing system, and beta-cell area/non–beta-cell area ratios were calculated.

* $P < .05$ vs vehicle-treated pair-fed *db/db* mice by analysis of variance followed by Fisher paired least squares difference.

** $P < .01$ vs vehicle-treated pair-fed *db/db* mice by analysis of variance followed by Fisher paired least squares difference.

† $P < .05$ vs vehicle-treated *+m/+m* control mice by analysis of variance followed by Fisher paired least squares difference.

†† $P < .01$ vs vehicle-treated *+m/+m* control mice by analysis of variance followed by Fisher paired least squares difference.

modulates hormonal concentrations in the pancreas of *db/db* mice. First, the number and total area of islets were measured by observation of HE-stained sections of the pancreas from BDNF- and vehicle-treated pair-fed *db/db* mice. The number of islets in the vehicle-treated pair-fed *db/db* mice was significantly higher than in normal control *+m/+m* mice (Table 1). However, there was no significant difference in the number of islets between BDNF- and vehicle-treated pair-fed *db/db* mice (Table 1). The total area of islets in the pancreas of vehicle-treated pair-fed *db/db* mice was significantly larger than in normal control *+m/+m* mice, and BDNF administration did not change the total area of islets in *db/db* mice, compared with vehicle (Table 1).

Because there was no significant change in the number and total area of islets between BDNF- and vehicle-treated pair-fed *db/db* mice, immunostaining analyses of pancreatic sections from *db/db* mice were performed after 4-week administration of BDNF or vehicle. Immunostaining using an anti-insulin antibody indicated that only a few insulin-positive beta cells were present in pancreatic islets from vehicle-treated pair-fed *db/db* mice compared with normoglycemic control *+m/+m* mice (Fig. 3, upper panels). In contrast, the area of insulin-positive beta cells in BDNF-treated *db/db* mice was larger than that in vehicle-treated pair-fed *db/db* mice and comparable to the area in *+m/+m* control mice (Fig. 3, upper panels). The beta-cell areas in pancreatic islets of BDNF- and vehicle-treated pair-fed *db/db* mice were also measured: the mean area of beta cells in 50 islets from vehicle-treated pair-fed *db/db* mice was smaller than in normal control *+m/+m* mice (Table 2), and BDNF treatment significantly increased the beta-cell area in pancreatic islets of *db/db* mice, compared with vehicle (Table 2).

Further identification of non–beta cells was performed by immunostaining using an anti-glucagon or an antisomatostatin antibody. An abnormal distribution of non-beta cells in pancreatic islets from vehicle-treated pair-fed *db/db* mice was observed (Fig. 3, middle and bottom panels). Non–beta cells in pancreatic islets from BDNF-treated *db/db* mice and from normal control *+m/+m* mice were found to be located in the periphery of pancreatic islets (Fig. 3, middle and bottom panels). The non–beta-cell area in pancreatic islets of BDNF- or vehicle-treated pair-fed *db/db* mice was also quantified: the mean area of non-beta cells in 50 islets from BDNF-treated *db/db* mice was significantly smaller than in vehicle-treated pair-fed *db/db* mice (Table 2). Taken together, these findings indicate that BDNF treatment significantly increased the ratio of the beta-cell area to

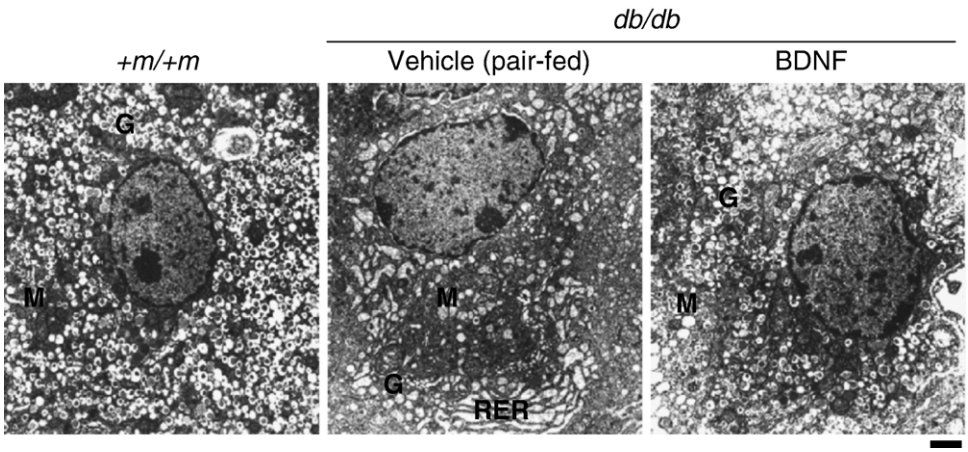

Fig. 4. Effect of BDNF on ultrastructure of pancreatic beta cells. Brain-derived neurotrophic factor (10 mg/kg) or vehicle was administered daily to *db/db* mice for 4 weeks. At the end of treatment, pancreatic tissues were excised and fixed with 2.5% glutaraldehyde and 2% osmic acid, then dehydrated and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead acetate. Each section was observed by electron microscopy. Representative pictures of pancreatic beta-cell sections of age-matched vehicle-treated *+m/+m* mice, vehicle-treated pair-fed *db/db* mice, or BDNF-treated *db/db* mice are shown (bar = 1 μm). G indicates Golgi apparatus; M, mitochondria; RER, rough endoplasmic reticulum.

non–beta-cell area in pancreatic islets of *db/db* mice compared with vehicle (Table 2).

3.4. Effect of BDNF administration on the ultrastructure of pancreatic beta cells

Ultrastructural changes are observed in pancreatic beta cells of diabetic mice compared with normoglycemic mice [16]. Electron microscopic analysis of pancreatic beta cells was performed to examine the effect of BDNF on the ultrastructure of pancreatic beta cells in *db/db* mice after the 4-week treatment period. The number of secretion granules, which presumably contain insulin, in beta cells from vehicle-treated pair-fed *db/db* mice was found to be markedly lower than in normal control *+m/+m* mice (Fig. 4). Enlargement of mitochondria, destruction of mitochondrial cristae, development of the Golgi apparatus, flattening of the rough endoplasmic reticulum (RER), and an increase in the amount of RER in electron microscopic images of pancreatic beta cells from vehicle-treated pair-fed *db/db* mice were observed compared with *+m/+m* mice (Fig. 4). All these observations indicate that protein synthesis and secretion are enhanced in pancreatic beta cells of vehicle-treated pair-fed *db/db* mice. The number of secretion granules increased in beta cells from BDNF-treated *db/db* mice, and the mitochondria, the Golgi apparatus, and the RER normalized to a level comparable to that in *+m/+m* mice (Fig. 4).

4. Discussion

We have previously demonstrated that administration of BDNF increases the insulin concentration in the pancreas of *db/db* mice compared with ad libitum–fed vehicle-treated *db/db* mice [10]. Because BDNF reduces food intake in obese hyperphagic diabetic animals, the alteration in the insulin concentration in the pancreas might be due to hypophagic effects of BDNF. Therefore, in the current study, a pellet pair-feeding apparatus was used to synchronize the food intake of vehicle-treated *db/db* mice with that of BDNF-treated *db/db* mice; this approach allowed a clear demonstration that BDNF administration increases the insulin concentration in the pancreas of *db/db* mice independently of its hypophagic action (Fig. 2B). Because BDNF treatment did not reduce the plasma insulin concentration in *db/db* mice compared with pair-fed controls (Fig. 2A), the increase in the pancreatic insulin concentration with BDNF treatment is unlikely to be due solely to a decrease in insulin secretion from pancreatic beta cells.

Our previous studies have shown that repetitive administration of BDNF improves insulin sensitivity in *db/db* mice by enhancing insulin-stimulated phosphatidylinositol 3-kinase activity in liver, skeletal muscle, and interscapular brown adipose tissue [17]. Thiazolidinediones (TZDs) are insulin-sensitizing agents that have also been reported to increase the pancreatic insulin concentration in *db/db* mice [16]. Therefore, BDNF and TZDs may increase the pancreatic insulin concentration by ameliorating insulin resistance in peripheral tissues, leading to reduced demands for excessive insulin secretion and preventing pancreatic exhaustion. Further analyses have demonstrated that TZDs increase the islet mass and the ratio of the beta-cell area to the non–beta-cell area, in addition to enhancing beta-cell granulation in *db/db* mice [16,18]. In the current study, BDNF also increased the ratio of the beta-cell area to the non–beta-cell area and enhanced beta-cell granulation in *db/db* mice (Table 2, Fig. 4), but did not increase the islet mass (Table 1). Thiazolidinediones are high-affinity ligands for peroxisome proliferator–activated receptor γ (PPARγ), which is expressed in beta cells of both rodents and humans [19,20], whereas the BDNF receptor, trkB, is not expressed in beta cells [21]. Beta cell–specific PPARγ-deficient mice exhibit abnormalities in islet mass, suggesting that PPARγ plays a critical physiologic role in beta-cell proliferation [22]. These previous findings and the results from this study indicate that TZDs regulate pancreatic beta-cell function, at least in part through a direct effect on beta-cell proliferation via PPARγ activation, whereas BDNF modulates pancreatic beta-cell function through a different mechanism.

The BDNF receptor, trkB, is expressed not only in the central and peripheral nervous systems, but also in some nonneuronal tissues, including pancreatic alpha cells [21]. Hanyu et al [23] have reported that BDNF decreases glucagon secretion in isolated mouse pancreatic islets without affecting insulin secretion. Therefore, it is possible that BDNF directly modulates the function of pancreatic alpha cells, and in the current study, BDNF treatment significantly decreased the pancreatic glucagon concentration compared with vehicle treatment (Fig. 2C). Moreover, BDNF suppresses hepatic glucose production in a hyperinsulinemic-euglycemic clamp using Zucker fatty rats [24]. Because an increase in glucagon levels causes elevated hepatic glucose production [25-27], reduction of the pancreatic glucagon concentration is likely to contribute to the hypoglycemic action of BDNF in *db/db* mice.

Brain-derived neurotrophic factor enhances energy expenditure and has many actions similar to those of the anorexic protein, leptin [10,17,28]. Leptin is an adipocyte-derived satiety factor that acts on the hypothalamus and leads to a significant reduction in body weight because of its ability to decrease food intake and increase energy expenditure [29]. Leptin directly inhibits insulin secretion in pancreatic islets and also reduces insulin secretion in vivo through the sympathetic nervous system [30,31]. The leptin receptor, Ob-Rb, is expressed in pancreatic beta cells [32,33]; in contrast, the BDNF receptor, trkB, is not expressed in pancreatic beta cells and BDNF does not inhibit insulin secretion in pancreatic islets [23,34]. However, BDNF has pleiotropic actions through the central nervous system, where trkB is abundantly expressed, and further analyses are required to determine whether BDNF has an effect on pancreatic function through the nervous system in a similar manner to leptin.

In summary, our results show that BDNF prevents exhaustion of the pancreas in *db/db* mice by maintaining the histologic cellular organization of beta cells and non-beta cells in pancreatic islets, and restoring the level of insulin-secreting granules in beta cells. These effects are likely to slow the progression of type 2 diabetes mellitus, indicating that BDNF may have potential as an antidiabetic agent.

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