Brain-derived neurotrophic factor in VMH as the causal factor for and therapeutic tool to treat visceral adiposity and hyperleptinemia in type 2 diabetic Goto–Kakizaki rats

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INTRODUCTION

The Goto–Kakizaki (GK) rat was established as an animal model for type 2 diabetes by selecting Wistar rats which exhibited glucose intolerance (Goto et al., 1976) and has been widely used to investigate the mechanisms of glucose intolerance and complications of hyperglycemia (Yagihashi et al., 1982; Picarel-Blanchot et al., 1996; Moreira et al., 2007). It has been reported that glucose intolerance in GK rats is mainly caused by reduced β-cell mass and impaired glucose-induced insulin secretion in β-cell (Portha et al., 1991). Glucose intolerance of GK rats is also thought to be partly due to impaired insulin sensitivity (Farese et al., 1994).

Our particular concern in this study is how impaired insulin sensitivity develops and is sustained in GK rats. We previously reported that impaired insulin sensitivity and increased visceral fat mass occurred in parallel in young adult (6–12 weeks) GK rats (Maekawa et al., 2006a). The result indicated a possibility that the increased visceral fat mass induced impairment of insulin sensitivity. In young adult (6–12 weeks), the increased fat mass was initiated by hyperphagia which was caused by enhanced neuropeptide Y (NPY) mRNA expression and impaired intracellular signaling of leptin in the hypothalamic arcuate nucleus (ARC) (Maekawa et al., 2006a). In GK rats, it has been also reported that insulin sensitivity is impaired not only at young adults but at middle-aged adults (Ndisang and Jadhav, 2009). Middle-aged GK rat in our colony (22–35 weeks) exhibited hyperleptinemia and higher mesenteric fat weight, while hyperphagia and overexpression of NPY mRNA in ARC were no longer observed (Maekawa et al., 2006a). Therefore, it is speculated that the long-lasting fat accumulation and hyperphagia in GK rats could provide an effective treatment of visceral obesity, hyperleptinemia and leptin resistance in type 2 diabetes.
hyperleptinemia at middle-age are due to decreased energy expenditure.

In this study, we focused on the relationship of brain-derived neurotrophic factor (BDNF) to fat accumulation and plasma leptin level in GK rat at middle-aged adult. In addition to well-defined role of BDNF in the regulation of development, survival, differentiation and synaptic plasticity in the nervous system (Tapia-Arancibia et al., 2004), it is also implicated in feeding behavior and energy balance (Noble et al., 2011; Rios, 2013). Heterozygous BDNF mutant mice and brain-specific BDNF knockout mice, in which the BDNF gene is selectively deleted in the brain after birth, showed increased food intake and body weight (Lyons et al., 1999; Kernie et al., 2000; Rios et al., 2001). Intracerebroventricular (icv) BDNF injection markedly reduced body weight in several obesity models by reducing appetite (Pelleymounter et al., 1995) and increasing energy expenditure (Nakagawa et al., 2000). In the hypothalamus, BDNF-expressing neurons are mainly localized in ventromedial hypothalamic nucleus (VMH) and paraventricular nucleus (PVN) (Noble et al., 2011). Since BDNF knockout in medial basal hypothalamus containing VMH induced hyperphagia and obesity, the BDNF neurons in this region is thought to be critical for regulating energy metabolism (Unger et al., 2007). BDNF neurons of VMH possibly project to the neurons expressing corticotrophin-releasing hormone (CRH) in PVN, since the CRH neurons express TrkB, a receptor for BDNF, and icv injection of BDNF increases CRH mRNA (Toriya et al., 2010). BDNF injection decreased respiratory quotient and increased rectal temperature, and these effects were antagonized by simultaneous treatment with α-helical CRH9–41, a CRH receptor antagonist (Toriya et al., 2010). These results suggested that the projection of BDNF neurons to CRH neurons in PVN plays a critical role in energy expenditure.

It has been reported that the BDNF expression in hypothalamus is regulated by several factors, which include neurotransmitters such as melanocortins (Nicholson et al., 2007; Vaneski and Xu, 2013), metabolic factors such as leptin (Komori et al., 2006), insulin and glucose (Unger et al., 2007), and environmental cues such as stress and environmental enrichment (Cao et al., 2010). In addition, BDNF level in hypothalamus is altered in obesity and/or diabetes models such as db/db, agouti yellow (Xu et al., 2003) and SF-1 knockout mice (Tran et al., 2003). These reports suggest that metabolic changes in obesity and diabetes result in and/or result from the reduction of BDNF expression.

Here, we found that BDNF expression is reduced specifically in VMH in GK rats at middle-age. We investigated the mechanism underlying the reduction of BDNF in VMH and examined whether BDNF supplementation induces lipolysis and ameliorates visceral obesity.

**MATERIALS AND METHODS**

**ANIMALS**

The GK rats purchased from Japan SLC (Shizuoka, Japan) were maintained by breeding in the Center for Experimental Medicine, Jichi Medical University. Adult male or pregnant Wistar rats were purchased from Japan SLC. The rats were housed under a controlled temperature (26°C) and photoperiod (12L:12D). The rats received pellet-type food (CE-2, Japan CLEA, Tokyo, Japan) and tap water *ad libitum*. The animal protocols were approved by the Jichi Medical School Institute of Animal Care and Use Committee and were in accord with the Japanese Physiological Society’s guidelines for animal care.

**mRNA EXTRACTION FROM BRAIN TISSUES AND cDNA SYNTHESIS**

A portion of hypothalamus in each rat was dissected using a brain slicer. The coronal slice of 2-mm thickness covering the anterior part of VMH to the posterior part of ARC was obtained. The tissues of the VMH and ARC were dissected with incisions, and homogenized with TRIzol (Invitrogen, Carlsbad, CA). Total RNAs were extracted following the protocol indicated by the manufacturer. DNase (1 U/10 μl RNA solution, Promega, Madison, WI) was added and the mixtures were incubated for 1 h at 37°C. Following the inactivation of DNase by heat, cDNA was synthesized from 2 μg total RNA with SuperscriptII Reverse Transcription Kit (Invitrogen) utilizing oligo(dT)20 primer.

**MIXED CULTURE OF CELLS FROM THE MEDIOBASAL HYPOTHALAMUS AND mRNA EXTRACTION**

The mediobasal hypothalamic tissue was isolated from the brain of 6-day-old pups of Wistar rats, followed by dissociation of neurons according to the procedures reported previously (Kohno et al., 2007) with slight modification. Briefly, brain slices in 1 mm-thickness were prepared, from which the tissues containing the VMH and ARC were obtained. The 5–6 dissected tissues were mixed and washed with 10 mM HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB) [(in mM): NaCl 129, NaHCO₃ 5.0, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 2.0, MgSO₄ 1.2 and HEPES 10 at pH 7.4] containing 10 mM glucose. Then they were incubated in HKRB supplemented with 20 units/ml papain (Sigma-Aldrich, St. Louis, MO), 0.015 mg/ml DNase, and 0.75 mg/ml BSA for 12 min at 36°C in a shaking water bath, followed by graded trituration. The cell suspension was incubated on ice for 5 min and a supernatant was centrifuged at 500 rpm for 5 min. The pellet was resuspended in the culture medium containing 50% minimal essential medium, 25% Hank’s balanced salt solution, 25% horse serum (#12360-038, #24020-117, #16050-122, Gibco BRL) and 10 mM glucose. The cells were distributed onto 96-well plates and short-term cultured. Each well-contained ~20% of cells removed from the brain of a pup. Three h after the treatment with 2-deoxy-d-glucose (2-DG) at various dosages, total RNA was extracted and cDNA was synthesized.

**mRNA MEASUREMENTS BY FLUORESCENCE REAL-TIME RT-PCR**

Quantification of mRNAs was carried out using ABI PRISM 7900-HT system (Applied Biosystems, Foster City, CA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level was measured as an internal control. PCR primer sets for amplification and TaqMan®probes with carboxyfluorescein in 3′end and carboxytetramethyl-rhodamine in 5′end were purchased from Sigma Genosys (Hokkaido, Japan) (Table A1). To detect GLUT8, SybGreen PCR was performed. Dividing each mRNA level by the GAPDH level resulted in a normalized mRNA value. The
fluorescence real-time PCR was performed by the method as described previously (Maekawa et al., 2006a). Dividing each mRNA level by the GAPDH level resulted in a normalized mRNA value.

Icv CANNULAE IMPLANTATION
Rats were anesthetized with Avertin (a mixture of 2,2,2-tribromoethanol (T4840-2, Sigma-Aldrich) and Tert-amyl alcohol (24,048-6, Sigma-Aldrich), 200 mg/kg, intraperitoneal). In a stereotaxic frame, a 23-gauged stainless steel guide cannula was inserted into the brain with the tip in the third cerebral ventricle, and secured to the skull with screws and cement. The cannula tip was located at 0.9 mm caudal to the bregma and 7.0 mm below the skull. After surgery, rats were allowed to recuperate for 7 days. Handling of the operated animals was performed for 10 min everyday. For injecting substances, the internal cannula was inserted into the guide cannula and injection was executed for 1 min under free-moving conditions.

IMMUNOHISTOCHEMISTRY
A chicken anti-human BDNF polyclonal antibody (1:100, G1641, Promega), a sheep anti-α-MSH antibody (1:30,000, AB5087, Chemicon, Temecula, CA), a biotinylated rabbit anti-chicken IgG (1:300, G2891, Promega), a biotinylated rabbit anti-sheep IgG (1:300, BA-6000, Vector Laboratories, Burlingame, CA) were used. Rats were intracerebroventricularly treated with 10 μl colchicine (100 μg/10 μl in distilled water). Forty-eight h after injection, rats were perfused transcardially with 100 ml of 2% paraformaldehyde in 50 mM phosphate buffer (PB), pH 7.5, followed by 50 ml of 50 mM phosphate-buffered saline under deep urethane anesthesia. Brains were removed, postfixed by the same fixative for 2 h, cryoprotected with 30% sucrose in 50 mM phosphate buffer (PB) for 2–3 days at 4°C, sectioned coronally at 40-μm thickness with a freezing microtome. The sections collected at every 160-μm interval were used. The localization of the target proteins was determined as described previously (Maekawa et al., 2006b).

Icv BDNF INJECTION, PLASMA HORMONE AND METABOLITES LEVELS
Wistar and GK rats were intracerebroventricularly injected with BDNF (15 μg/5 μl saline/head) or vehicle once a day for 6 days. Plasma samples were obtained at the day before injection, the 6th, 10th, 21st day after injection. Blood was withdrawn from tail vein under unanesthetized condition. Glucose level was determined by a conventional blood glucose-measuring device (Glucocard, Arkray, Kyoto, Japan). Intraperitoneal glucose tolerance test (IPGTT) was performed under overnight fasting conditions. Glucose (1 g/kg body weight) was injected and then blood samplings from the tail vein were performed up to 3 h. For hormonal assay, plasma was immediately separated. Plasma leptin and insulin concentrations were measured using ELISA kits for leptin and insulin, respectively (#200728 and #200718, Morinaga Institute of Biological Science, Yokohama, Japan). Plasma nonesterified free fatty acid (NEFA) concentration was measured using an assay kit for NEFA (#279-75401 NEFA C-test Wako, Osaka, Japan).

MEASUREMENTS OF FOOD INTAKE AND BODY WEIGHT COMBINED WITH PAIR-FEEDING
Food intake over 24 h was calculated by weighing the remaining food pellets and body weight was measured between 11:00 and 12:00 h every day. For pair-feeding experiment, the amount of food consumed by the BDNF-treated group over the course of 24 h was measured at 11:00 h, and a corresponding amount of pellets was given to the pair-fed group over a 24-h period.

MEASUREMENT OF FAT MASSES
At the completion of each experiment, interscapular, epididymal, mesenteric, and perirenal fat were dissected and weighed. Fat masses were calculated as percentage of body weight.

DATA ANALYSES
All data are expressed as mean ± SEM. The number of animals used is indicated in the parenthesis. One-Way ANOVA with Holm’s post-hoc test was used for Figures 3D, 4, 5A,B, 6, 7A,B, Table 2. Two-Way ANOVA with Tukey’s post-hoc test was used for Figures 1B, 8, and Table 1. Other data was analyzed by Student’s t-test with Microsoft Excel 2008 for Mac (Microsoft, Redmond, WA). All statistical analyses except Student’s t-test were performed using R (The R Foundation for Statistical Computing, Vienna, Austria). p < 0.05 was considered significant.

RESULTS

METABOLIC INDICES OF GK RATS AT 26 WEEKS OF AGE
Casual blood glucose level in GK rat was significantly higher than that in Wistar rat at 11 weeks and it increased further at 26 weeks (Figure A1A). Intraperitoneal glucose tolerance test in GK rats revealed that glucose intolerance progressed at 24 weeks, compared to 14 weeks of age (Figure A1B). Mesenteric and perirenal fat weights were larger in GK rats than in Wistar rats at 11 and 26 weeks of age (Table 1).

REDUCED BDNF EXPRESSION IN VMH OF GK RATS AT 26 WEEKS OF AGE
By comparison between Wistar and GK rats at 11 and 26 weeks of age, BDNF mRNA level examined by real-time RT-PCR was found to be reduced in VMH of GK rat specifically at 26 weeks of age (Figure 1A, p < 0.05). Furthermore, immunohistochemistry revealed marked reduction in the number of BDNF expressing neurons selectively in VMH but not in PVN and nucleus tractus solitarius (NTS) of GK rat at 26 weeks of age (Figures 1B–J, p < 0.05 in B).

MECHANISM UNDERLYING THE REDUCED BDNF EXPRESSION IN VMH OF GK RATS
α-MSH, melanocortin-4 receptor (MC4R), insulin, leptin and glucose have been reported to affect BDNF expression. Hence, we examined possible involvement of these factors in reduction of BDNF expression in GK rat. Based on the previous report showing a link between BDNF and α-MSH (Xu et al., 2003; Nicholson et al., 2007), we examined a possible alteration of melanocortin system in GK rats. By immunohistochemistry, localization of α-MSH-immunoreactive neurons in the ARC of GK rats was identical to that in control Wistar rats (Figures 2A,B). The cell numbers of α-MSH-immunoreactive neurons in the ARC were...
Table 1 | Interscapular, epididymal, mesenteric and perirenal fat weights (% body weight) in control Wistar and diabetic GK rats at 11 and 26 weeks of age.

| Age   | Strain | No. | Interscapular | Epididymal | Mesenteric | Perirenal |
|-------|--------|-----|---------------|------------|------------|-----------|
| 11 weeks | Wistar   | 6   | 0.09 ± 0.01   | 1.47 ± 0.08 | 0.63 ± 0.05 | 0.93 ± 0.04 |
|       | GK      | 5   | 0.21 ± 0.02*  | 1.04 ± 0.05* | 1.08 ± 0.04* | 1.78 ± 0.11* |
| 26 weeks | Wistar   | 5   | 0.08 ± 0.02   | 1.52 ± 0.11 | 0.89 ± 0.05 | 1.55 ± 0.14 |
|       | GK      | 6   | 0.12 ± 0.01   | 1.16 ± 0.07* | 1.27 ± 0.12* | 1.97 ± 0.11* |

*p < 0.05 vs Control Wistar rats (Two-Way ANOVA with Tukey's post-hoc test).

not different between GK and Wistar rats (Figure 2C). Similarly, no difference in MC4R mRNA expression was found in the ARC and VMH in GK rats (Figure 2D).

Next, the possible association of insulin and/or leptin with BDNF reduction was examined. We tested whether the supplementation of insulin would rescue BDNF reduction in GK rats. GK rats were treated with intraperitoneal administration of insulin (1 U/kg body weight) twice a day for 3 days, which failed to restore the BDNF mRNA levels in the VMH (Figure 2E). To examine the acute effect of insulin to increase BDNF mRNA level in the VMH, single icv injection of insulin (10 mU/10 μl saline) to Wistar rats was performed. However, it did not increase the BDNF mRNA expression (Figure 2F). Icv administration of leptin (5 μg, twice a day for 3 days) in Wistar rats under fasting condition failed to alter BDNF mRNA level (Figure 2G).

Since transcription of BDNF in the VMH has been reported to be regulated by glucose (Unger et al., 2007), we examined the mRNA expression of genes related to the glucose utilization in the VMH of GK rats using real-time RT-PCR. There was no difference in GLUT4, GLUT8 and glucokinase mRNA expressions between Wistar and GK rats (Figure 3A). In contrast, the GLUT2 mRNA level was significantly reduced at 26 weeks compared to 11 weeks of age (Figure 3B). To test the possibility that reduction of glucose utilization affects BDNF mRNA level in the VMH, 2-DG (5 mg/10 μl saline), an inhibitor of glycolysis, was administered icv to Wistar rats. 2-DG markedly reduced BDNF mRNA level in the VMH as well as in hippocampus at 2 h after treatment (Figure 3C). In a parallel in vitro experiment, bath application of 2-DG to primary cultured neurons of medial basal hypothalamus including the VMH dose-dependently reduced BDNF mRNA expression (Figure 3D). These results indicate that lowered glucose utilization in the VMH directly reduces BDNF mRNA expression.

EFFECT OF BDNF TREATMENT ON FOOD CONSUMPTION, BODY WEIGHT, GLUCOSE TOLERANCE, AND NEFA LEVEL IN GK RATS

Daily food intake was significantly lowered both in Wistar-BDNF and GK-BDNF groups during BDNF treatment (Figure 5A). After termination of treatment, the food intake in Wistar-BDNF and GK-BDNF groups rebounded to the same level as that in GK-Vehicle group (Figure 5A). BDNF treatments also reduced body weights in the rats of Wistar-BDNF and GK-BDNF groups (Figure 5B). After termination of treatment, the body weight of the BDNF-treated groups rebounded to the level close to that before BDNF treatment (Figure 5B). Although the blood glucose level in GK-BDNF level did not change during BDNF treatment, after termination of treatment it became lower than that in GK-Vehicle group, demonstrating the late-onset effect of BDNF on glucose control (Figure 6). To investigate the mechanism how BDNF lowered the casual glucose level in GK-BDNF group, we performed IpGTT and measured glucose and insulin levels during IpGTT (Figure 7). Blood glucose levels during IpGTT in GK-Vehicle and GK-BDNF groups were markedly higher than that in Wistar-BDNF groups at Day 0 (before treatment), Day 7 (immediately after termination of treatment), and Day 11 (after termination of treatment) (Figure 7A). Blood glucose level during IpGTT at Day 7 tended to increase, rather than decrease, in GK-BDNF group. The higher glucose level at Day 7 in GK-BDNF group is probably due to the lowered insulin level at Day 7 (Figure 7B). These results taken together suggest that BDNF treatments lowered casual blood glucose level by changing insulin sensitivity but not by improving insulin secretion in GK rats subjected to treatment with saline revealed higher plasma leptin level during experiment (GK-Vehicle group, Figure 4). By contrast, the treatments with BDNF significantly decreased plasma leptin level in both Wistar and GK rats (Wistar-BDNF and GK-BDNF groups, respectively) at the timing of termination of treatment (Day 6) and at 4 days after termination of treatment (Day 10) (p < 0.01, Figure 4). Plasma leptin level at 15 days after termination of treatment (Day 21) returned to the level before treatment in GK rats of GK-BDNF group, while it was still low in Wistar-BDNF group. At Day 21, weights of fat mass in all groups were measured (Table 2). The weight of mesenteric fat in BDNF-treated GK rats (GK-BDNF group) was significantly lower than that in saline-treated GK rats (GK-Vehicle group, p < 0.05), although it was higher than that in BDNF-treated Wistar rats.
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**FIGURE 1** | Expressions of BDNF mRNA and BDNF-immunoreactive cells in Wistar and GK rats at 11 and 26 weeks of age. (A) BDNF mRNA levels in VMH of Wistar and GK rats. Significant reduction of BDNF mRNA level was found in GK rats at 26 weeks of age (*p < 0.05, Student's t-test). (B) At 26 weeks, significant reduction of the number of BDNF-immunoreactive neurons was found in VMH, but not PVN and NTS, of GK rats (*p < 0.05, Two-Way ANOVA with Tukey's post-hoc test). This reduction in VMH was not different at 11 weeks. (C–J) Representative images of BDNF-immunoreactive neurons in Wistar rats (C,E,G,I) and GK rats (D,F,H,J) at 26 weeks of age. Broken lines in each image indicate the outline of PVN (C,D) and VMH (E,F). No apparent difference was found in the PVN (C,D) and NTS (G,H). By contrast, the number of BDNF-immunoreactive neurons in the VMH of GK rats (F) was fewer than that of Wistar rats (E). At higher magnification, the difference between Wistar (I) and GK (J) rats was obvious. III; the third ventricle, IV; the fourth ventricle, cc; central canal. Scale bar = 500 μm (C–H) or 100 μm (I and J).

To examine whether exogenous BDNF treatment changes plasma NEFA level in GK rats, we measured plasma NEFA level in fed and 12h-fasted conditions. In GK-Vehicle group, plasma NEFA level was higher in fasted condition at all time points measured (Figure 8). In GK-BDNF and Wistar-BNDF groups, plasma NEFA level was significantly increased both in fed and fasted conditions at Day 6(fed)/7(fasted) and returned to pre-treatment level at Day 10 (fed)/11(fasted) (Figure 8), indicating...
We examined plasma leptin levels in GK-BDNF and GK-Vehicle pair-fed groups. In GK-BDNF group, at Day 6 and 10 plasma leptin levels were markedly reduced compared to the fasting Wistar rats. However, at Day 10 it increased to a level significantly higher than that in GK-BDNF group, indicating that exogenous BDNF counteracted visceral adiposity and hyperleptinemia via two modes of action: in acute phase via anorexigenic action and in late long-lasting phase via food intake-independent mechanisms.

**DISCUSSION**

In this study, we found that middle-aged GK rats show abdominal fat accumulation and hyperleptinemia, reduction in BDNF mRNA expression and protein levels specifically in VMH, and reduction in GLUT2 mRNA in VMH. Pharmacological blockade of glucose utilization in vivo and in vitro reduced BDNF expression in VMH, suggesting that glucose availability positively regulates BDNF expression in VMH. In rescue experiment, BDNF supplementation for 6 days ameliorated hyperleptinemia and higher adiposity in a long-lasting manner. These results reveal that reduction of BDNF expression due to decreased GLUT2 expression and glucose utilization in VMH is linked to visceral fat accumulation and hyperleptinemia in GK rats.

**FAT ACCUMULATION AND GLUCOSE INTOLERANCE IN MIDDLE-AGED GK RATS**

It has been reported that glucose intolerance in human type 2 diabetes progresses with age (Gong and Muzumdar, 2012). Major cause for this progression is insulin resistance due to genetic and/or environmental factors. Especially, visceral fat accumulation is of particular concern as a causal factor to induce insulin resistance (Pouliot et al., 1992). Our breeding colony of GK rats displayed these characteristic features common for the type 2 diabetes. First, hyperglycemia and glucose intolerance progressed markedly at 24 weeks of age compared to 14 weeks (Figure A1). Second, the visceral adiposity in mesenteric and perirenal fat pads in GK rats was greater at 26 weeks of age than at 11 weeks. Third, progression of hyperglycemia paralleled with progression of adiposity. These findings suggested that higher visceral adiposity is related to the progression of glucose intolerance and that GK rat is a suitable animal model to study interaction of glucose intolerance and visceral adiposity in type 2 diabetes.

**REDUCTION OF BDNF EXPRESSION IN VMH OF GK RATS AT MIDDLE AGE**

BDNF in the brain plays a critical role in regulating feeding and metabolism. The BDNF neurons expressed in VMH and PVN have been reported to contribute to the regulation of feeding and metabolism (Noble et al., 2011). We found that the GLUT2 mRNA level in VMH was significantly reduced in GK rats. Previous studies in GK rats

**COMPARISON OF PLASMA LEPTIN LEVELS BETWEEN GK-BDNF AND GK-VEHICLE PAIR-FED GROUPS**

We examined plasma leptin levels in GK-BDNF and GK-Vehicle pair-fed groups (Figure 9). In GK-Vehicle pair-fed group, GK rats were treated with saline and the amount of food supplied was controlled to the same level as consumed by GK-BDNF group. In GK-BDNF group, at Day 6 and 10 plasma leptin levels were markedly reduced (Figure 9). In GK-Vehicle pair-fed group, plasma leptin level was lowered to the same level as in GK-BDNF group at Day 6. However, at Day 10 it increased to a level significantly higher than that in GK-BDNF group, indicating that exogenous BDNF counteracted visceral adiposity and hyperleptinemia via two modes of action: in acute phase via anorexigenic action and in late long-lasting phase via food intake-independent mechanisms.

**FIGURE 2** Lack of alterations of melanocortines and effects of insulin and leptin on BDNF mRNA expression in GK rats. (A,B) Representative images of α-MSH-immunoreactive neurons in ARC of Wistar (A) and GK (B) rats; III; the third ventricle, Scale bar = 500 μm. (C) Numbers of α-MSH-immunoreactive neurons in Wistar and GK rats. (D) MC4R mRNA expressions in ARC and VMH were not different between Wistar and GK rats. (E) Repeated intraperitoneal injection of insulin (1 U/kg body weight, twice a day for 3 days) to the fasting Wistar rats had no significant effect on BDNF mRNA level in VMH. (F) Single icv injection of insulin (10 mU) in Wistar rats did not alter BDNF mRNA levels in VMH. (G) Repeated icv injection of leptin (5 μg, twice a day for 3 days) to the fasting Wistar rats had no significant effect on BDNF mRNA levels in VMH of Wistar rats.
reported reductions of GLUT1 in the retina (Fernandes et al., 2004), GLUT2 in the pancreatic islets (Ohneda et al., 1993) and GLUT4 in the heart (Desrois et al., 2004). Glucose uptake in the skeletal muscle was also reported to be impaired in GK rats (Krook et al., 1997). As a causal factor of GLUT reduction, it has been implicated that over-activation of hexosamine pathway by hyperglycemia decreases the expression level of GLUT2 in the pancreatic β-cells, eventually leading to the inhibition of glucose-stimulated insulin secretion and induction of apoptosis in β-cells (Yoshikawa et al., 2002). The down-regulation of GLUT2 is expected to suppress the glucose uptake and utilization in a specific subset of neurons presumably including BDNF-expressing neurons, which could suppress BDNF mRNA in the VMH of GK rats. In support of this speculation, icv injection of 2-DG (5 mg/10 μl) or saline (Sal) BDNF mRNA expression in VMH and hippocampus (Hp) was significantly reduced by 2-DG (*p < 0.05, Student's t-test). (D) Treatment with 2-DG for 3 h dose-dependently suppressed BDNF mRNA level in cultured mediobasal hypothalamic cells. The effects of 12 and 16 mM 2-DG were significant (*p < 0.05, One-Way ANOVA with Holm's post-hoc test).
Table 2 | Effect of BDNF on interscapular, epididymal, mesenteric and perirenal fat weights (% body weight) in GK rats.

| Strain | Treatment | No. | Interscapular Weight of Fat pad (% BW) | Epididymal | Mesenteric | Perirenal |
|--------|-----------|-----|--------------------------------------|------------|------------|----------|
| GK     | Vehicle   | 6   | 0.15 ± 0.01                          | 1.02 ± 0.11| 1.14 ± 0.08| 1.18 ± 0.14|
| GK     | BDNF      | 5   | 0.17 ± 0.02                          | 0.80 ± 0.07| 0.80 ± 0.09*| 0.97 ± 0.17|
| Wistar | BDNF      | 4   | 0.05 ± 0.01*                         | 0.99 ± 0.08| 0.55 ± 0.06*| 0.56 ± 0.07*|

Fat weights of saline-treated GK rats (GK-Vehicle group, 5 µl saline/head, n = 6), BDNF-treated GK rats (GK-BDNF group, 15 µg/5 µl saline/head, n = 5) and BDNF-treated Wistar rats (Wistar-BDNF group, 15 µg/5 µl saline/head, n = 4) at Day 21 after the beginning of injections were examined. Interscapular: *p < 0.05 vs. other groups, Mesenteric: *p < 0.05 vs. GK-Vehicle, Perirenal: *p < 0.05 vs. GK-Vehicle, One-Way ANOVA with Holm’s post-hoc test.

In middle-aged GK rats with reduced level of BDNF in VMH, hyperphagia was not found as shown in previous study (Maekawa et al., 2006a). On the other hand, it has been reported that the reduced level of BDNF in medial basal hypothalamus by conditional BDNF gene knockout causes hyperphagia (Unger et al., 2007). What causes the difference between these phenotypes? It has been reported that the BDNF neurons in VMH regulate various physiological functions such as feeding, energy expenditure, and blood glucose control (Noble et al., 2011). It could be speculated that each function is assigned to a specific subgroup of BDNF-expressing neurons in VMH. Since the BDNF-expressing neurons at the level up to 30% of that in Wistar rats persisted in middle-aged GK rats, such subgroup of BDNF neurons might play a role in preventing hyperphagia whereas the subgroup might take no part in the regulation of lipolysis. This possibility should be validated in future study.

CENTRAL BDNF SUPPLEMENTATION AMELIORATED HIGHER ADIPOSITY IN GK RATS

It has been reported that lesioning the VMH induces not only hyperphagia but also a marked visceral fat accumulation in Wistar (Bray and Nishizawa, 1978) and GK rats (Yoshida et al., 1996), and that the loss of neurons in the VMH induces dysfunction of lipolysis. The neurons in VMH are known to project to various...
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FIGURE 7 | Intraperitoneal glucose tolerance test before and after BDNF treatment in GK rats. BDNF treatment did not attenuate glucose intolerance (A) and impaired insulin release (B) during glucose tolerance test in GK rats throughout experiment. (A) $p < 0.05$ vs. GK-Vehicle and GK-BDNF (One-Way ANOVA with Holm’s post-hoc test). (B) $p < 0.05$ vs. GK-Vehicle in Day 0 (30 min), Day 7 (15 min), $p < 0.05$ vs. GK-BDNF in Day 7 (15 min, 30 min) and 11 (15 min), One-Way ANOVA with Holm’s post-hoc test.

brain areas. In our previous report using Wistar rats (Maekawa et al., 2006b), only 19% of BDNF neurons of VMH project to midbrain central gray, a region known to be innervated by VMH neurons. PVN is one of possible candidates for primary synaptic transmission of VMH BDNF neurons. Our previous report suggested that TrkB receptor, a neurotrophin receptor specific for BDNF, is expressed in CRH neurons of PVN and icv BDNF injection increases the CRH mRNA in PVN (Toriya et al., 2010). Although there is no direct evidence that BDNF neurons of VMH are connected to CRH neurons in PVN, these results obtained by physiological experiments indirectly support the possibility that the VMH BDNF neurons project to CRH neurons in PVN. It has been demonstrated that CRH neuron in PVN regulates both sympathetic tone and hypothalamic-pituitary-adrenal axis and that CRH neurons positively control lipolysis (Yada et al., 2012). Our previous report demonstrated that lypolytic effect of BDNF was concontrated by simultaneous treatment with CRH receptor antagonist, suggesting that the lypolytic effect of BDNF is mediated by CRH release from CRH neurons in PVN (Toriya et al., 2010). Thus, our present and previous results taken together suggest a possibility that the reduction of BDNF in VMH impairs lipolysis via reducing CRH release in GK rats. Other than CRH release, TrkB and CRH receptor in hypothalamus including PVN might be changed concomitantly with the change in CRH release in GK rats. Such possibilities should also be taken into account when elucidating the etiology of visceral fat accumulation in GK rats.

BDNF has been reported to regulate the binding of cAMP response-element binding protein (CREB) and coactivator proteins to CRH promoter and thereby positively control transcription of CRH (Jeanneteau et al., 2012). Activation of phospholipase Cγ might mediate the BDNF-TrkB signaling by triggering Ca$^{2+}$ increase, activating adenylate cyclase and subsequently increasing cAMP concentration (Ji et al., 2005). On the other hand, BNDF works not only as a neurotransmitter but as a regulator of synaptic structural plasticity (Yoshii and Constantine-Paton, 2010).
microRNA which binds to BDNF mRNA (Gao et al., 2010). Study of such proteins to BDNF reduction should be examined in future study. Has been also reported that glycolysis inhibition by 2-DG injection NADH-binding co-repressor CtBP to BDNF promoter. Notably, it NRSF, a transcriptional repressor, has been reported to recruit the expression is neuron-restrictive silencer factor (NRSF). Another possible protein to link glucose metabolism to BDNF Jeong et al., 2011), and by suppressing expression of specific expression by deacetylating MeCP2 (Zocchi and Sassone-Corsi, deacetylase. It has been reported that SIRT1 controls BDNF control of feeding and energy metabolism.

**FUTURE PERSPECTIVE**

The molecular mechanisms connecting lower glucose metabolism to BDNF reduction remained unclear in this study. One of key proteins that link glucose metabolism to BDNF expression is SIRT1, a metabolic sensor by working as a NAD⁺-dependent deacetylase. It has been reported that SIRT1 controls BDNF expression by deacetylating MeCP2 (Zocchi and Sassone-Corsi, 2012), by enhancing CREB-TORC1 transcriptional activity (Jeong et al., 2011), and by suppressing expression of specific microRNA which binds to BDNF mRNA (Gao et al., 2010). Another possible protein to link glucose metabolism to BDNF expression is neuron-restrictive silencer factor (NRSF). The NRSF, a transcriptional repressor, has been reported to recruit the NADH-binding co-repressor CtBP to BDNF promoter. Notably, it has been also reported that glycolysis inhibition by 2-DG injection reduces BDNF transcription by NRSF-CtBP-dependent change of histone modification (Garriga-Canut et al., 2006). Involvements of such proteins to BDNF reduction should be examined in future study.

In this study, we found that BDNF treatments reduce fat mass and lower casual blood glucose level by changing insulin sensitivity. However, BDNF could not restore the insulin secretion in GK rats. Therefore, to improve the glycemic control in type-2 diabetes more effectively, the simultaneous treatment with BDNF and the drug having a function to alleviate impaired insulin secretion might be required. Therapeutic potential of the combination of BDNF with the drug enhancing insulin secretion against diabetes should be investigated in future study.

**CONCLUSION**

In this study, we present the following scenario to explain how the higher visceral adiposity continues in GK rats until middle-aged adult. (1) The hyperglycemia and/or other diabetes-associated factors suppress the GLUT2 expression to lower the glucose availability in VMH. (2) The reduction of glucose availability leads to impair BDNF expression in VMH. (3) The reduced BDNF level attenuates lypolysis and accelerates glucose intolerance. Further studies are required to validate whether this scenario in GK rats is more generally applicable to other diabetes and/or obesity models.

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**FIGURE A1 | Glycemic profiles of GK rats.** (A) The blood glucose level under fed conditions in Wistar and GK rats at the age of 11 and 26 weeks. The blood glucose level in GK rats was significantly higher than that in Wistar rats at both 11 and 26 weeks (*p < 0.05 and **p < 0.01, respectively, Student’s t-test). The difference was greater at 26 weeks. (B) Profiles of IpGTT in GK rats at 14 and 24 weeks. One g/kg body weight glucose (0.1 g/ml in distilled water) was injected at 0 min and blood glucose was measured at 0, 30, 60, and 120 min after injection. The glucose intolerance was progressed in GK rats at 24 weeks of age (**p < 0.01, Student’s t-test).

**Table A1 | Primer sequences used for real-time RT-PCR.**

| mRNA (Abbreviation) | Genbank accession number (amplicon) | Primers | Probe (5’-FAM, 3’-TAMRA) |
|---------------------|-------------------------------------|---------|-------------------------|
| BDNF                | NM012513 (504-571)                 | Fwd: CCATAAGGACGCAGGACCTTGTAC Rvs: GAGGAGGCTCCAAAGGCACCTTT | TTCCCGGGTGATGCTCAGCAGT |
| MCRY4               | NM013099 (731-819)                 | Fwd: TGGCGAGGCTTCTACCATAAAGA Rvs: CAAGGTAATTGCGCCCTTCA | CAGGTTACCATCGACAGGGTG |
| GLUT2               | NM012879 (818-898)                 | Fwd: GTCCAGAAGCCCCAGAGCTTGTAC Rvs: CAAGGTAATTGCGCCCTTCA | TTCCCGGATCTCCTTCAAGGCTT |
| GLUT4               | NM012751 (818-907)                 | Fwd: CCCCCGATACCTCTACTACTTAC Rvs: GCATCAGACACATCAGCCAG | CGGCAGAAGAGAGTCTAAAGGCCT |
| GLUT8               | NM053494 (1049-1235)               | Fwd: TCAAGCTCCACCGGAGGCG Rvs: GAGGACGCCAGCCAGCCAG | TCAGGCTCGCCACACTGGCG |
| Glucokinase        | NM012565 (1330-1414)               | Fwd: CAAGCTGGCAGGGCT TTTAGGGT Rvs: TGGATCGATGAGGTTTCAG | Not used |