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Increased expression of adenylyl cyclase 3 in pancreatic islets and central nervous system of diabetic Goto-Kakizaki rats

A possible regulatory role in glucose homeostasis

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Abbreviations: AC3, Adenylyl cyclase 3; CNS, Central nervous system; GK, Goto-Kakizaki; RGS9, regulator of G protein signalling 9; T2D, type 2 diabetes

Adenylyl cyclase 3 (*AC3*) is expressed in pancreatic islets of the Goto-Kakizaki (GK) rat, a spontaneous animal model of type 2 diabetes (T2D), and also exerts genetic effects on the regulation of body weight in man. In addition to pancreatic islets, the central nervous system (CNS) plays an important role in the pathogenesis of T2D and obesity by regulating feeding behavior, body weight and glucose metabolism. In the present study, we have investigated *AC3* expression in pancreatic islets, striatum and hypothalamus of GK rats to evaluate its role in the regulation of glucose homeostasis. GK and Wistar rats at the age of 2.5 mo were used. A group of GK rats were implanted with sustained insulin release chips for 15 d. Plasma glucose and serum insulin levels were measured. *AC3* gene expression levels in pancreatic islets, striatum and hypothalamus were determined by using real-time RT-PCR. Results indicated that plasma glucose levels in Wistar rats were found to be similar to insulin-treated GK rats, and significantly lower compared with non-treated GK rats. *AC3* expression levels in pancreatic islets, striatum and hypothalamus of GK rats were higher compared with Wistar rats, while the levels were intermediate in insulin-treated GK rats. The *AC3* expression display patterns between pancreatic islets and striatum-hypothalamus were similar. The present study thus provides the first evidence that *AC3* is overexpressed in the regions of striatum and hypothalamus of brain, and similarly in pancreatic islets of GK rats suggesting that *AC3* plays a role in regulation of glucose homeostasis via CNS and insulin secretion.

Introduction

Type 2 diabetes (T2D) is a heterogeneous disorder characterized by hyperglycemia, which is mainly due to insulin deficiency and insulin resistance. T2D is often associated with obesity and the prevalence of both T2D and obesity increases with age.[1-3] The brain may play an important role in the regulation of feeding behavior, body weight and glucose metabolism. Therefore, identification of the involved genes and analysis of their biological effects in the central nervous system (CNS) may provide useful information to better understand the molecular mechanisms underlying T2D and obesity.[4-6]

Adenylyl cyclases (ACs or ADCYs) are enzymes that catalyze the synthesis of 3'-5'-cyclic adenosine monophosphate (cAMP) from ATP. cAMP is an important second messenger, which mediates downstream activity of protein kinase A and subsequently regulates insulin secretion in β-cells of pancreatic islets.[7-9] There are nine closely related isoforms of ACs (AC1–9) in mammals. All AC isoforms share a primary structure consisting of two transmembrane regions M1 and M2, each containing six predicted membrane-spanning helices and two cytoplasmic regions. AC3 has been characterized as one of the calcium-dependent isoforms.[10,11]

Goto-Kakizaki (GK) rat is a hereditary non-obese animal model of T2D, which exhibits a markedly reduced glucose-induced insulin release. This animal model has been commonly used in genetic and functional analyses of pancreatic hormones and glucose metabolism in relation to T2D.[12-15] A study from our laboratory has demonstrated that the *AC3* gene at mRNA levels is overexpressed in pancreatic islets of GK rat by using in situ hybridization.[16] In a genetic association study of the *AC3* gene in Swedish T2D patients and obese subjects, we found that the *AC3* genetic polymorphisms have protective effects in obesity

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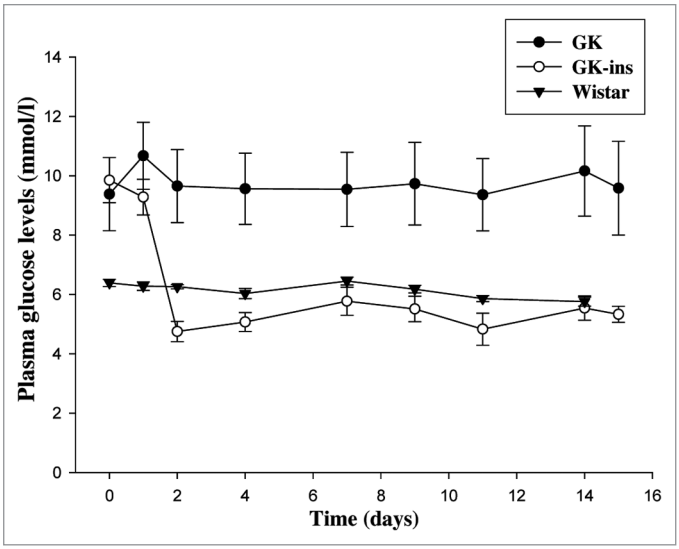

Figure 1. Serial measurement of plasma glucose levels in GK, insulin-treated GK and Wistar rats. n = 11, 11, and 14 for GK, insulin-treated GK, and Wistar rats, respectively. Data were means ± SE.

but are not associated with plasma glucose and insulin levels in the patients with T2D.[17] We have replicated the genetic association study in a Chinese population and confirmed that the *AC3* genetic polymorphisms are associated with decreased risk of obesity among adults.[18] In further support, Wang et al. have demonstrated that *AC3* knockout mice develop obesity when aging.[19]

Although data from our and other studies have implicated that AC3 may play an important role in the regulation of body weight,[20] some questions remain to be elucidated. First, whether the increased *AC3* expression found in pancreatic islets of GK rats is a primary defect or secondary to hyperglycemia. Second, whether *AC3* is expressed in CNS and thus may be implicated in the regulation of glucose metabolism and body weight. To address the questions, in the present study, we have applied insulin treatment to normalize the plasma glucose levels in GK rats and then assessed *AC3* expression with a specific gene expression assay not only in pancreatic islets, but also in hypothalamus and striatum regions of brain. Furthermore, ACs are regulated by G-proteins, being activated by Gαs[21] and inhibited by Gαi.[22] Regulators of G protein signaling (RGS) are a family of proteins, which regulate ACs either directly or by inactivating Gαs.[23] A recent study has demonstrated that mice deficient of *RGS9*, a member of RGS family, alike *AC3* knockout mice develop obesity.[24,25] The *RGS9* gene is highly enriched in the striatum region of brain. In order to ascertain whether *AC3* is expressed in the striatum region of brain and also to understand whether the *AC3* gene has an interaction with *RGS9* in brain, we have studied the *AC3* gene expressions in the dissected tissues of hypothalamus and striatum from *RGS9* knockout and wild-type mice.

Results

Plasma glucose levels and body weights were measured regularly in Wistar and GK rats with and without insulin treatment, whereas serum insulin levels in all animals were determined when they were sacrificed. In GK rats with treatment of sustained release insulin chips, plasma glucose levels were reduced from the second day and remained at similar levels as in Wistar rats until they were killed on the fifteenth day (**Fig. 1**).

Comparison tests of plasma glucose levels, body weight and serum insulin levels among Wistar, GK rats with and without insulin treatment at sacrifice were done. Data are represented in **Figure 2A, B and C** respectively. Plasma glucose levels in GK rats (9.6 ± 1.6 mmol/l) were significantly higher compared with Wistar (5.8 ± 0.1 mmol/l, p = 0.008) and insulin-treated GK rats (5.3 ± 0.3 mmol/l, p = 0.005) (**Fig. 2A**). Body weights of GK rats with (287.8 ± 3.2 g) and without insulin treatment (273.1 ± 3.3 g) were lower compared with Wistar rats (400.2 ± 8.6 g, p < 0.001) (**Fig. 2B**). Serum insulin levels in GK and Wistar rats were similar (38.4 ± 3.4 vs. 46.0 ± 7.3 μU/ml, p = 0.657) (**Fig. 2C**). As expected, serum insulin levels in insulin-treated GK rats were significantly higher compared with Wistar and GK rats without insulin treatment (68.4 ± 6.0 vs. 46.0 ± 7.3 and 38.4 ± 3.4 μU/ml, p = 0.035/0.006).

Insulin release was determined in batches of isolated pancreatic islets. In Wistar rats, insulin release at 3.3 and 16.7 mmol/l glucose was 47.6 ± 8.0 and 124.9 ± 14.3 μU/islet/h, respectively (p < 0.001). In the islets from GK rats without insulin treatment, insulin release at 3.3 and 16.7 mmol/l glucose was 23.2 ± 4.0 and 44.2 ± 10.0 μU/islet/h, respectively (p = 0.057), and in islets from insulin-treated GK rats, insulin release was 17.8 ± 3.2 and 45.5 ± 13.4 μU/islet/h, respectively (p = 0.059).

AC3 mRNA expression levels in pancreatic islets and regions of striatum/hypothalamus of brain were significantly different between the three groups of rats. **Figure 3A** indicates that *AC3* mRNA expression levels in pancreatic islet of Wistar, insulin-treated GK and GK rats were gradually increased (p = 0.018). However, a statistically significant difference between GK and Wistar rats was seen (p = 0.016) but not between insulin-treated GK and Wistar rats. **Figure 3B** shows a similar pattern of *AC3* expression in regions of striatum/hypothalamus of brain among these three groups of rats (p = 0.026) as seen in pancreatic islets. Similar to what we observed in the pancreatic islets, *AC3* mRNA expression in striatum/hypothalamus of GK rats without (p = 0.021) but not with insulin treatment was significantly increased compared with Wistar rats. *AC3* mRNA expression levels in tissues of liver and white fat were detectable but no significant difference among the groups of GK, insulin-treated GK and Wistar rats was observed (data not showed).

In order to ascertain whether *AC3* has interaction with *RGS9*, we examined the *AC3* expression in striatum and hypothalamus tissues dissected from *RGS9* knockout and wild-type mice because it has been shown that *RGS9* is enriched in striatum region of brain.[24,25] Results indicated that *RGS9* was expressed in striatum and hypothalamus regions of brain in wild-type mice but not in *RGS9*-deficient mice. However, *AC3* was similarly expressed in striatum and hypothalamus regions of both wild-type and *RGS9*-deficient mice (**Fig. 4**).

Discussion

We have investigated the *AC3* gene expression levels in non-treated GK, insulin-treated GK and Wistar rats. Our data not only confirm that *AC3* is expressed in pancreatic islets but also demonstrate that *AC3* is expressed in the striatum and hypothalamus regions of brain. In pancreatic islets and striatum-hypothalamus, the *AC3* mRNA expression levels in GK rats are increased compared with Wistar rats, and tended to be reduced in insulin-treated GK rats compared with GK rats without insulin treatment.

The previous study from our laboratory has observed that *AC3* is overexpressed in pancreatic islets of GK rats compared with Wistar rats by using in situ hybridization method.[16] In the present study, we have confirmed that *AC3* expression is upregulated in pancreatic islets of GK rats compared with Wistar rats with specific TaqMan gene expression analysis. Furthermore, we have demonstrated that *AC3* is expressed in the region of striatum-hypothalamus of brain. Since *AC3* is expressed in striatum and hypothalamus tissues from *RGS9* knockout mice, it seems unlikely that the *AC3* and *RGS9* genes interact. Our attempts to analyze the *AC3* gene expression at the protein levels with western blotting technique failed, mainly due to the high homologies of AC3 with other AC isoforms and the lack of specific high-affinity antibodies.[8]

We have applied insulin treatment to normalize blood glucose levels in GK rats for 15 d. Comparison analyses between Wistar, GK rats with and without insulin treatment provided us the opportunity to investigate whether overexpression of *AC3* is a primary defect or rather secondary to the diabetic state (hyperglycemia). Results from our experiments have demonstrated that the *AC3* expression patterns in pancreatic islets and the regions of striatum and hypothalamus are similar. In both pancreatic islets and striatum-hypothalamus of insulin-treated GK rats, the *AC3* expression levels were intermediate between GK and Wistar rats but not significantly different. This suggests that normalization of plasma glucose levels in GK rats with insulin treatment tended to normalize the augmented *AC3* mRNA expression. Notably, the normalization of glycemia did not restore glucose-stimulated insulin release in isolated islets. Furthermore, several reports have demonstrated that neuronal signaling, consisting of both afferent and efferent autonomic nerves, plays important roles in inter-organ metabolic communication and systemic homeostasis.[26,27] Based upon our observation of the similarity of *AC3* gene expression patterns in pancreatic islets and the regions of striatum-hypothalamus, we herein provide a hypothesis regarding the existence of a functional link between CNS and pancreatic islets via AC3 regulation.

We have recently demonstrated the *AC3* genetic polymorphisms are associated with obesity in Swedish and Chinese populations.[17,18] Wang et al. have developed an *AC3*-deficient mouse model which exhibit obesity mainly due to low locomotor activity, hyperphagia and leptin resistance.[19] Thus, AC3 appears to be of genetic and biological relevance in the regulation of body weight and glucose homeostasis.[20] In the present study, we have measured the body weights of Wistar and GK rats with and without insulin treatment. The body weights in both GK rats with and without insulin treatment are similar but significantly lower than in Wistar rats. Taking together with the information from genetic association studies in human subjects and experimental studies with animals, we suggest that AC3 may have a

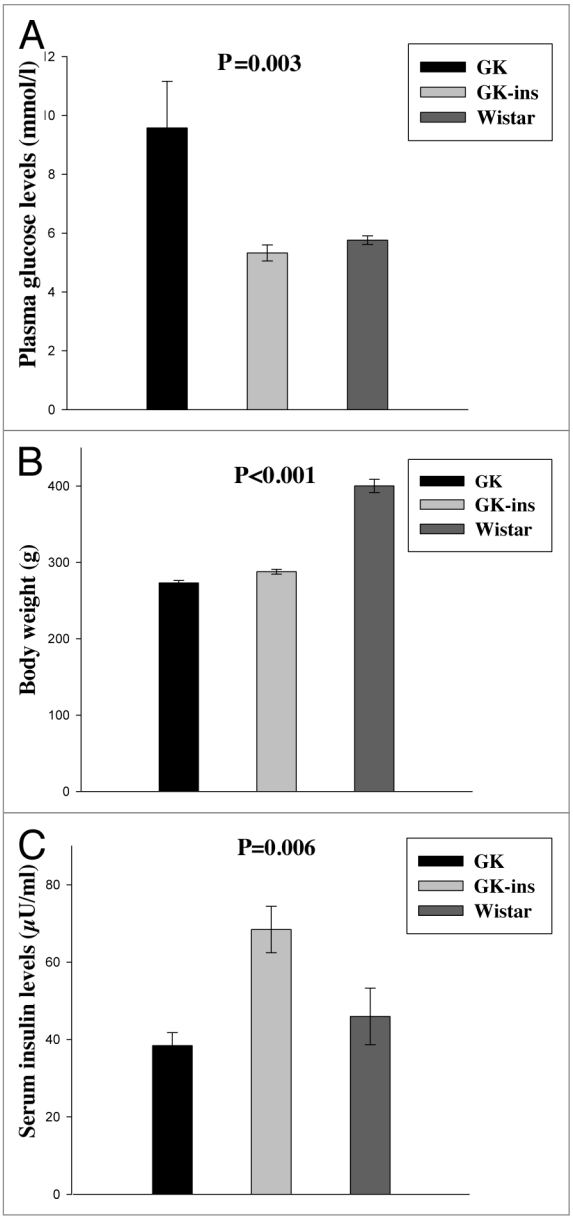

Figure 2. Plasma glucose levels (**A**), body weight (**B**) and serum insulin levels (**C**) of GK, insulin-treated GK and Wistar rats at sacrifice. (**A**) Plasma glucose levels in GK rats (n = 11) were higher compared with GK rats with insulin treatment (n = 11, p = 0.005) and Wistar rats (n = 14, p = 0.008). There was no difference between insulin-treated GK and Wistar rats; (**B**) Body weights in Wistar rats were higher than in GK rats with and without insulin treatment (p < 0.001). Body weights of GK and insulin-treated GK rats were similar; (**C**) GK rats with insulin treatment had increased serum insulin levels compared with GK rats without insulin treatment (p = 0.006) and also to Wistar rats (p = 0.035). Data were means ± SE; p values were from ANOVA analyses among GK, insulin-treated GK and Wistar rats. Pairwise comparisons were performed with Tukey post-hoc test.

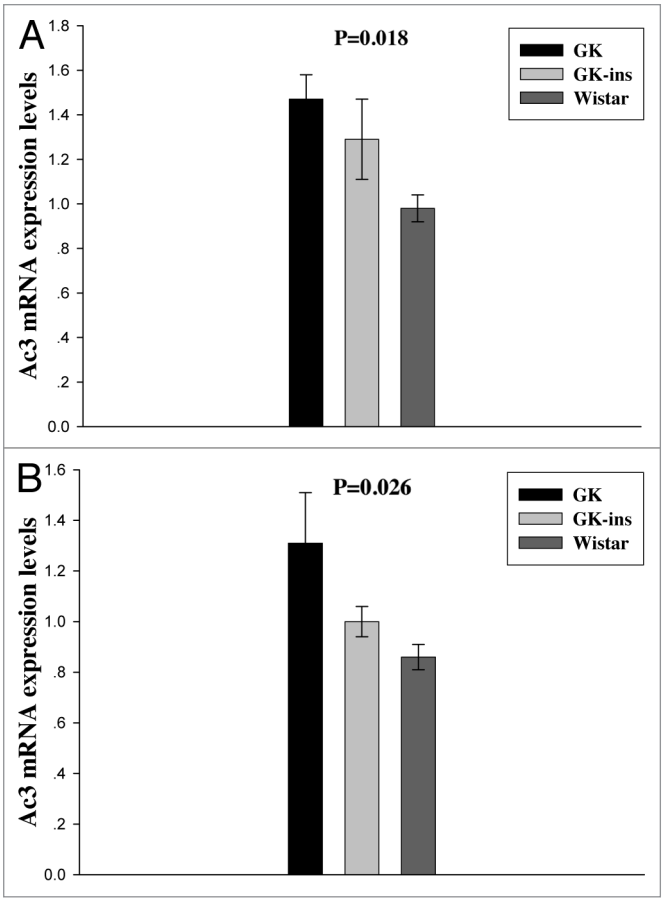

Figure 3. *AC3* mRNA expression levels in pancreatic islets (**A**) and striatum-hypothalamus regions of brain (**B**). *AC3* mRNA expression levels in both pancreatic islets and striatum-hypothalamus regions of brain of GK rats were significantly upregulated compared with Wistar rats (p = 0.016, 0.021, respectively). *AC3* expression levels in both pancreatic islets and striatum-hypothalamus regions of brain of insulin-treated GK rats were relatively decreased compared with GK rats without insulin treatment but still higher than in Wistar rats. Data were means ± SE; P-values were from ANOVA analyses among GK, insulin-treated GK and Wistar rats. Pairwise comparisons were performed with Tukey post-hoc test.

primary role on the regulation of body weight. This primary role of AC3 may be controlled or influenced by CNS.

In conclusion, the present study provides evidence that *AC3* is overexpressed in the regions of striatum and hypothalamus of brain and similarly in pancreatic islets of GK rats. It is also suggested that *AC3* plays a role in regulating glucose homeostasis via insulin secretion and CNS but is unlikely to interact with *RGS9* in the brain.

Material and Methods

Animals. A total of 22 male GK rats, at the age of 2.5 mo, were obtained from our colony at Karolinska University Hospital, and 14 age-matched male Wistar rats from a local breeder (B&K Universal) were used as controls. Of the GK rats, 11 were implanted with sustained insulin release chips containing 26 ± 2 mg microcrystallized bovine insulin palmitic acid (LinShin Inc.) for 15 d. All rats had free access to food. Plasma glucose levels and body weights were measured during the 15-d period. The samples of tissues, including pancreatic islets, liver and white fat, were collected, and the regions of striatum and hypothalamus in brain were dissected when the rats were sacrificed on the 15th day. All tissue samples were stored with RNAlater (Ambion) at −80°C. Serum insulin levels were determined using radioimmunoassay (RIA).

In order to investigate whether *AC3* has co-localization and interaction with *RGS9* in the striatum and hypothalamus regions of brain, we have studied *AC3* and *RGS9* gene expressions in the dissected striatum and hypothalamus tissue samples from *RGS9* knockout and wild-type mice (n = 7 in each group). All mice were provided by University of Rhode Island and the procedures were done with the approval from the animal care and use committee in this University. Generation and genotyping of the $RGS9^{-/-}$ mice has been described previously by Chen et al.[24] Heterozygous crosses were used as the breeding strategy to generate the wild-type ($RGS9^{+/+}$) and *RGS9* knockout ($RGS9^{-/-}$) littermates used for this study.

Isolation and incubation of rat islets. Pancreata were removed after retrograde injection in the pancreatic duct of 24 mg or 9 mg collagenase in 10 ml Hank's solution (SVA) in GK or Wistar rats, respectively. The pancreata were then incubated at 37°C for 24 min and homogenized by suction 5–10 times through a 14 G × 3 1/8" needle into a syringe. Three washing steps in Hank's solution followed, after which we made histopaque gradient by mixing the pancreata with 5 ml 1119 and 5 ml 1077 histopaque solutions (Sigma-Aldrich). Then, we slowly added 5 ml 1077 histopaque solution upon the mixture and 5 ml Hank's solution on the top; thus, three layers were formed. The homogenate was centrifuged at 2000 rpm for 20 min. Following centrifugation, islets were collected from the border of the upper two layers, namely, 1077 histopaque and Hank's layers. The islets were then transferred to Hank's medium. Part of islets was collected and stored in RNAlater solution for total RNA extraction. The remaining islets, in batches of three islets each, were incubated for 60 min at 37°C water-bath, slowly shaking in 300 μl Krebs-Ringer bicarbonate (KRB) buffer solution with 3.3 or 16.7 mmol/l glucose. After incubation, 200 μl was aspirated to a new tube, kept in the freezer at −20°C till insulin release has been measured using (RIA).

RNA extraction and real time RT-PCR. In rats, total cellular RNA was extracted using RNeasy mini kit, following the manufacturer's protocol for tissues (Qiagen). To minimize the risk of RNA degradation, the samples were kept on ice when not performing the extraction. All working surfaces and tools were cleaned with RNase Away solution (Sigma) prior to use. Reverse-transcription (RT) for cDNA from mRNA samples was performed using QuantiTect reverse transcription kit (Qiagen). In mice, the tissues of striatum and hypothalamus were dissected using visual landmarks and total cellular RNA was isolated using Trizol reagents according to manufacturer's instructions (Invitrogen). Two striatal tissue samples were obtained from each mouse (1 per hemisphere), whereas one hypothalamus enriched sample was obtained from each mouse. RNA was reverse

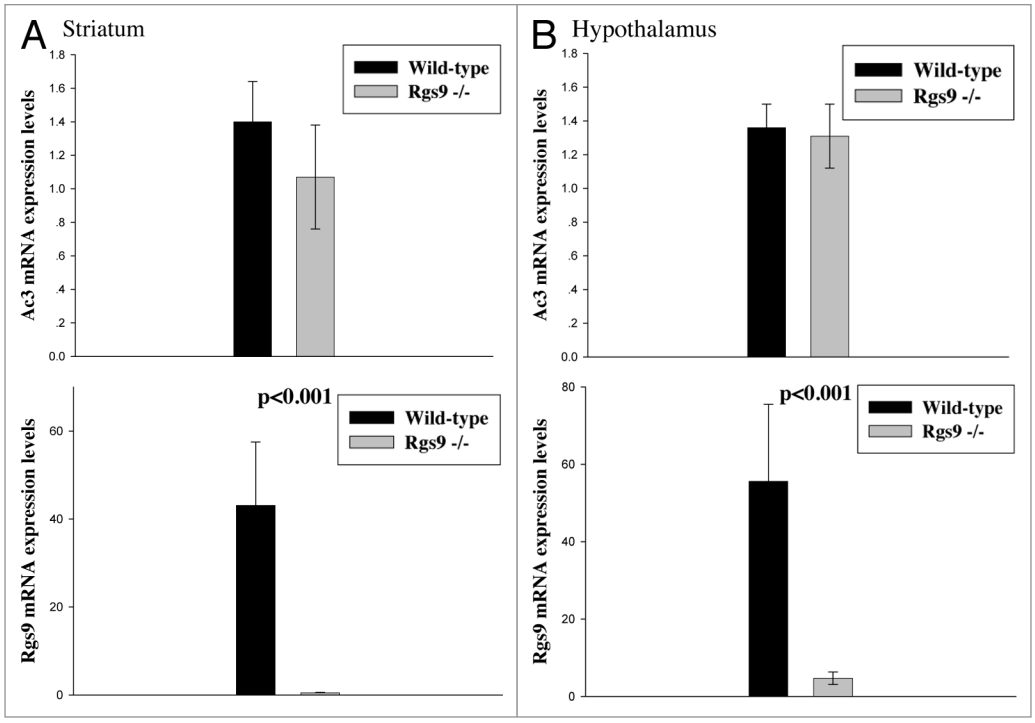

Figure 4. *AC3* (above) and *RGS9* (below) mRNA expression levels in striatum and hypothalamus regions of brain of *RGS9* knockout and wild-type mice. *AC3* mRNA expression levels in both striatum and hypothalamus regions of brain were similar in *RGS9* knockout and wild-type mice. *RGS9* was expressed in striatum and hypothalamus regions of brain of wild-type but not in *RGS9* knockout mice ($p < 0.001$). Data were means ± SE; p values were from Student's t-test.

transcribed with M-MLV reverse transcriptase enzyme (Sigma-Aldrich) and the random hexamer primer at 37°C for 50 min.

Real time RT-PCR was performed with TaqMan gene expression assays specifically for the *AC3* gene in rat and mouse (Applied Biosystems) and with an ABI 7300 real-time PCR system (Applied Biosystems). The ID numbers of TaqMan gene expression assays for study of the *AC3* gene in rat and mouse were Rn01469282_m1 and Mm00460371_m1, respectively. Sequence information of the primers and probes is available in the database of TaqMan gene expression assays (Applied Biosystems). Briefly, the amplicon length is 85 bp and covers the boundary between exons 19 and 20. In *RGS9* knockout and wild-type mice, TaqMan gene expression assay for study of the *RGS9* gene (ID number is Mm01250425_m1) was also used. The assays of 18s (the amplicon length is 61 bp) and β-actin (91 bp) were chosen as the reference genes for normalization. Negative controls including genomic DNA and water blanks were included on each plate. The probes of all TaqMan gene expression assays were labeled with 6'-carboxy-fluorescein (FAM) as a reporter dye and TAMRA as a quencher dye. Amplifications were performed using the 5'-nuclease TaqMan method with a two-step PCR protocol (95°C for 10 min, followed by 35 cycles of 95°C for 15 sec and 60°C for 1 min) in an ABI 7300 real-time PCR system (Applied Biosystems). Experiments were replicated on at least two occasions. Gene expression data were analyzed using the relative quantification method based upon the standard curve.

Statistical analysis. Data were analyzed using the programs of PASW (formerly SPSS, version 18) and SigmaPlot (version 2001). The differences in continuous variables among the groups of Wistar rats and GK rats with and without insulin treatment were evaluated with ANOVA and Tukey's HSD (Honestly Significant Difference) post-hoc test. For comparison analyses between *RGS9* knockout and wild-type mice, Student's t-test was used. Data were given as means ± SE. p value of 0.05 or less was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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