ATP-sensitive K^+ channels in the hypothalamus are essential for the maintenance of glucose homeostasis

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Glucose-responsive (GR) neurons in the hypothalamus are thought to be critical in glucose homeostasis, but it is not known how they function in this context. Kir6.2 is the pore-forming subunit of K_{ATP} channels in many cell types, including pancreatic β-cells and heart. Here we show the complete absence of both functional ATP-sensitive K^+ (K_{ATP}) channels and glucose responsiveness in the neurons of the ventromedial hypothalamus (VMH) in *Kir6.2*$^{-/-}$ mice. Although pancreatic α-cells were functional in *Kir6.2*$^{-/-}$, the mice exhibited a severe defect in glucagon secretion in response to systemic hypoglycemia. In addition, they showed a complete loss of glucagon secretion, together with reduced food intake in response to neuroglycopenia. Thus, our results demonstrate that K_{ATP} channels are important in glucose sensing in VMH GR neurons, and are essential for the maintenance of glucose homeostasis.

K_{ATP} channels are found in many tissues, including skeletal and smooth muscle, heart, pancreatic β-cells, pituitary, and brain[1]. These channels are thought to regulate various cellular functions such as hormone secretion[2], excitability of neurons[3] and muscles[4], and cytoprotection during ischemia[5,6] by coupling cell metabolism to membrane potential[1]. The K_{ATP} channels in pancreatic β-cells are critical metabolic sensors that determine glucose-responsive membrane excitability in the regulation of insulin secretion[7]. In the brain, K_{ATP} channels have been found in many regions, including substantia nigra[8], neocortex[9], hippocampus[10] and hypothalamus[11]. In the VMH, the GR neurons increase their firing rate in response to elevation of extracellular glucose levels[12,13]. Although K_{ATP} channels are present in GR neurons[11,14], their molecular identity and functional role are unclear. In addition, the contribution of GR neurons to systemic glucose homeostasis is not known.

The K_{ATP} channel is an octameric protein consisting of two subunits: the pore-forming inward rectifier K^+ channel member Kir6.1 or Kir6.2, and the sulfonylurea receptor SUR1 or SUR2 (SUR2A, SUR2B or possibly other SUR2 splice variants)[15–18]. Whereas pancreatic β-cell K_{ATP} channels comprise Kir6.2 and SUR1, cardiac K_{ATP} channels consist of Kir6.2 and SUR2A[15–17]. For different neuronal populations, all possible coexpression patterns of Kir6.1 or Kir6.2 and SUR1 or SUR2A have been reported[10,19–21].

We generated *Kir6.2*-deficient mice (*Kir6.2*$^{-/-}$)[22], in which both glucose-responsive and sulfonylurea-induced insulin secretion were defective[22]. During the course of our study, we found that recovery from hypoglycemia was impaired in *Kir6.2*$^{-/-}$ mice, suggesting that the secretion of counter-regulatory hormones in response to low glucose levels is impaired. Because the hypothalamus, especially the VMH, is important in the control of counter-regulatory hormones[23–25], we investigated the molecular composition and functional role of K_{ATP} channels in the hypothalamic GR neurons of *Kir6.2*$^{-/-}$ and wild-type mice (*Kir6.2*$^{+/+}$).

Results

Response of glucagon and epinephrine to hypoglycemia

We found that recovery from systemic hypoglycemia induced by insulin injection was severely impaired in *Kir6.2*$^{-/-}$ mice (Fig. 1a), suggesting a deficiency in the secretion of counter-regulatory hormones such as glucagon and catecholamines. We then evaluated the counter-regulatory hormone secretion. Although epinephrine secretion in response to insulin-induced hypoglycemia was similar in *Kir6.2*$^{-/-}$ and *Kir6.2*$^{+/+}$ mice (Fig. 1b), the glucagon secretion was markedly reduced in *Kir6.2*$^{-/-}$ mice (Fig. 1c). Because K_{ATP} channels comprising Kir6.2 are present not only in the insulin-secreting β-cells of pancreatic islets[26,27] but also in other endocrine cells of the islets including glucagon-secreting α-cells[28,29], we examined glucagon secretion from isolated pancreatic islets. The glucagon secretion in response to change from high (16.7 mM) to low (1 mM) glucose concentration was similar in *Kir6.2*$^{-/-}$ and *Kir6.2*$^{+/+}$ mice (Fig. 1d). In addition, the glucagon response to a synthetic choline ester, carbachol (50 μM), was not impaired but was somewhat enhanced in

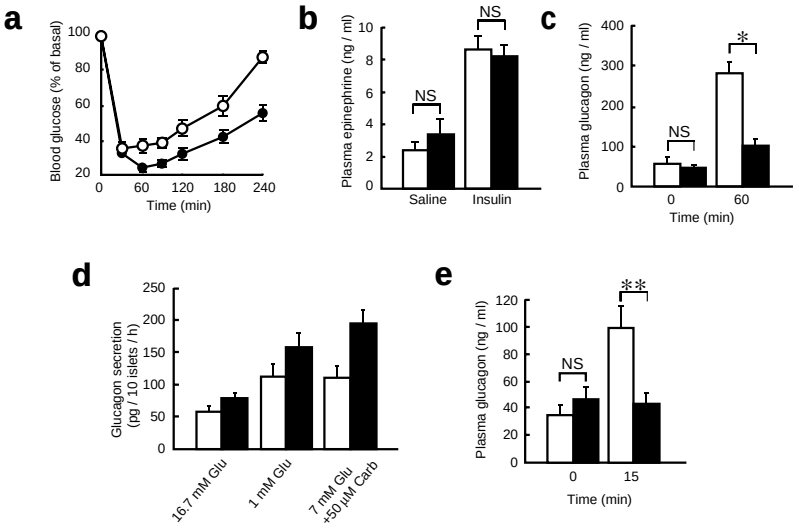

Fig. 1. Blood glucose levels, epinephrine and glucagon secretion in *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ mice. (**a**) Changes in blood glucose levels after exogenous insulin injection. Human insulin (0.5 IU/kg) was injected intraperitoneally to conscious male mice. Results are expressed as percent of initial blood glucose concentration. Open circles, *Kir6.2*$^{+/+}$ mice; filled circles, *Kir6.2*$^{-/-}$ mice (n = 7 for each). (**b**) Epinephrine secretion induced by insulin-induced hypoglycemia. Plasma epinephrine levels were measured 60 min after injection of either human insulin (1 IU/kg) or saline (n = 5 for each). (**c**) Glucagon secretion by insulin-induced hypoglycemia. Hypoglycemia was induced as described in Fig. 1b. Plasma glucagon levels were measured before and 60 min after insulin injection (n = 13 for each). *p < 0.0001. (**d**) Glucagon secretion from pancreatic islets. Glucagon secretion from the islets incubated with Krebs–Ringer bicarbonate buffer containing 16.7 mM glucose (Glu), 1 mM glucose or 7 mM glucose plus 50 μM carbachol (Carb) is shown. The results were obtained from 3–4 independent experiments (n = 11–17). (**e**) Glucagon secretion *in vivo* by intracerebroventricular administration of 2DG. Plasma glucagon levels were measured before and 15 min after the injection of 2DG (1 mg/body; n = 6). **p < 0.02. For (**b**–**e**), open columns and filled columns represent *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ mice, respectively. The values are means ± s.e.m. NS, not significant.

Kir6.2$^{-/-}$ compared to *Kir6.2*$^{+/+}$ mice. These results suggest that both the glucose sensing by the α-cells and the response of the α-cells to autonomic input[30] remain unaffected in *Kir6.2*$^{-/-}$, and that the primary defect in these mice is upstream of the α-cells.

Response of glucagon to neuroglycopenia

In the brain, neuroglycopenia stimulates glucagon secretion through activation of autonomic neurons. In the hypothalamus, 2-deoxyglucose (2DG) induces neuroglycopenia[31], thereby stimulating glucagon secretion[24,32]. To determine the effect of 2DG on glucagon secretion in *Kir6.2*$^{-/-}$, 2DG was injected into the third ventricle. The administration of 2DG produced an increase in glucagon secretion in *Kir6.2*$^{+/+}$ but not in *Kir6.2*$^{-/-}$ mice (Fig. 1e).

Glucose responsiveness of VMH neurons

Because the VMH possesses the highest density of GR neurons and is involved in glucagon secretion during hypoglycemia[23–25], we examined the electrophysiological properties of VMH neurons in *in vitro* brain slices from *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ mice. We distinguished three different neuronal populations, based on their spontaneous firing rates and their distinct subthreshold rebound behavior (Fig. 2a), as has been reported previously[13]. Type A neurons quickly resumed spiking at the end of hyperpolarizing current injections (Fig. 2a, top). In contrast, type B neurons showed rebound calcium spikes (Fig. 2a, middle), whereas type C neurons displayed a prominent delay in repolarization from hyperpolarized membrane potentials (Fig. 2a, bottom). There were no differences in the relative abundance of these three VMH cell types in brain slices from *Kir6.2*$^{-/-}$ and

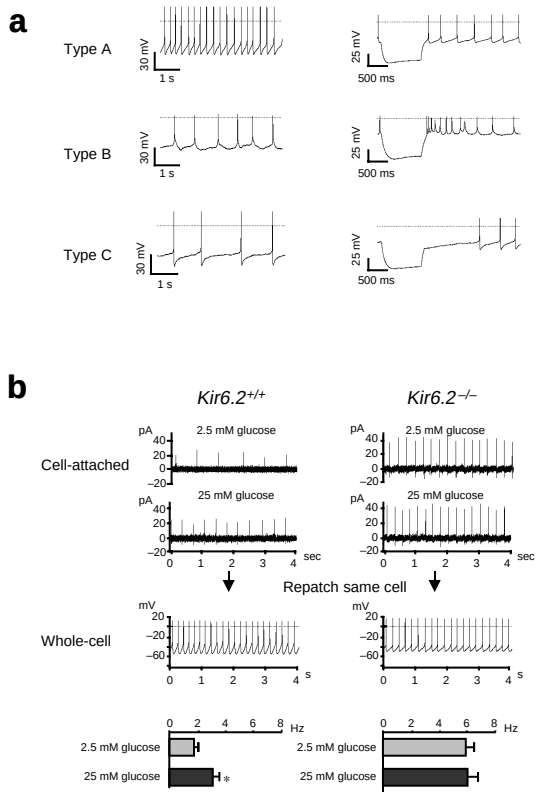

Fig. 2. Electrophysiological properties and glucose responsiveness of VMH neurons. (**a**) Three different types of VMH neurons were identified by their electrophysiological properties. Representative current-clamp recordings of spontaneous activity (left) and of responses to 50 pA injection of hyperpolarizing current (right) are shown. (**b**) VMH neurons of *Kir6.2*$^{-/-}$ are defective in glucose sensing. Shown are cell-attached recordings of spontaneous activity in response to an increase in extracellular glucose concentrations from 2.5 to 25 mM (top panels). After cell-attached recordings, the same neurons were repatched for whole-cell recordings to identify the neuronal cell type (middle). Repatching of identified GR neurons in *Kir6.2*$^{+/+}$ demonstrated that they were either type A or type C neurons. VMH neurons of *Kir6.2*$^{-/-}$ already exhibited spontaneous activity with a higher frequency compared to *Kir6.2*$^{+/+}$ of 2.5 mM glucose, and no further increase in activity was observed in response to increased glucose concentration (top right). Bottom, summary cell firing rates in low and high concentrations of glucose. The values are means ± s.e.m. Twenty-four percent of VMH neurons in *Kir6.2*$^{+/+}$ but none in *Kir6.2*$^{-/-}$ responded to increased glucose concentrations. *p < 0.01.

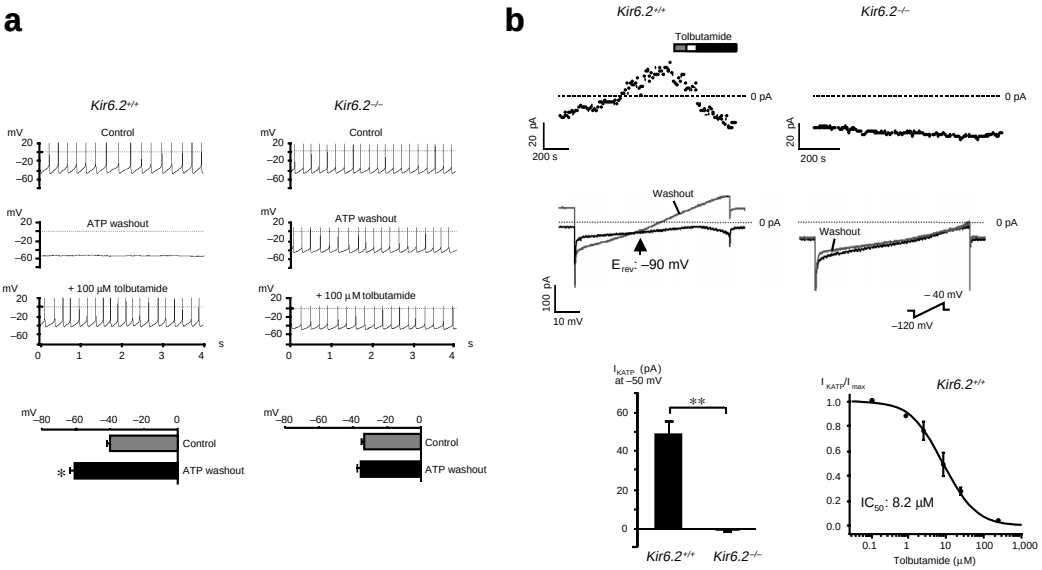

Fig. 3. Characterization of K_{ATP} channels in VMH neurons of *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$. (**a**) Standard whole-cell current-clamp recordings of VMH neurons in *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$. Traces show spontaneous activity of membrane potential in 25 mM extracellular glucose concentration (top), after 15 min dialysis with ATP-free pipette (middle), and after ATP washout plus application of 100 μM tolbutamide (bottom). Bottom, summary of changes in membrane potential after ATP washout in *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$. *$p < 0.001$. (**b**) Standard whole-cell voltage-clamp recordings of K_{ATP} channel currents in VMH neurons. Top, current responses at –50 mV, each plotted 10 s during dialysis with ATP-free pipette solution. Application of tolbutamide is indicated by the boxes (gray, 3 μM; white, 30 μM; black, 300 μM). Middle, current responses to voltage ramps from –120 mV to –40 mV before and after ATP washout (E_{rev} = reversal potential of the activated current). Bottom left, summary of outward K^+ currents activated by ATP washout at –50 mV. *$p < 0.0001$. Bottom right, dose–response curve for tolbutamide inhibition of normalized K_{ATP} whole-cell currents activated by dialysis with ATP-free pipette solution (I_{KATP}/I_{max}) in VMH neurons of *Kir6.2*$^{+/+}$ ($n = 4$). The values are means ± s.e.m. The line represents the fit of the mean data by a Hill equation with an IC_{50} of 8.2 μM and a Hill coefficient of 1.0.

Kir6.2$^{+/+}$ mice, suggesting the structural integrity of the VMH in *Kir6.2*$^{-/-}$ (*Kir6.2*$^{+/+}$, type A, 47%, $n = 16/34$; type B, 32%, $n = 11/34$; type C, 21%, $n = 7/34$; *Kir6.2*$^{-/-}$, type A, 45%, $n = 21/46$; type B, 35%, $n = 16/46$; type C 20%, $n = 9/46$).

To examine glucose sensing by VMH neurons under physiological metabolic conditions, we recorded neuronal activity in the cell-attached patch configuration at different extracellular glucose concentrations (Fig. 2b, top), and repatched the neurons for subsequent cell-type identification in the whole-cell mode (Fig. 2b, middle). Consistently with previous studies[33,34], 24% ($n = 8/33$) of *Kir6.2*$^{+/+}$ VMH neurons showed an approximately twofold increase in spontaneous discharge rate (1.8 ± 0.4, $p < 0.001$) in response to an increase in glucose from 2.5 mM (1.8 ± 0.2 Hz) to 25 mM glucose (3.2 ± 0.3 Hz, Fig. 2b, bottom left). Repatching these glucose responsive neurons demonstrated that they were either type A or type C neurons. In contrast, none of the *Kir6.2*$^{-/-}$ VMH neurons, including all identified type A or type C neurons, displayed changes in their spiking frequency in response to increased glucose (2.5 mM glucose, 6.0 ± 0.6 Hz; 25 mM glucose, 6.1 ± 0.7 Hz, $n = 27$; Fig. 2b, bottom right). In addition, *Kir6.2*$^{-/-}$ VMH neurons displayed higher discharge rates at low glucose concentrations than those of *Kir6.2*$^{+/+}$. Taken together, these results strongly suggest that Kir6.2-containing K_{ATP} channels are essential for glucose sensing in VMH neurons.

Functional property of the K_{ATP} channel in VMH neurons

To ascertain the presence of K_{ATP} channel currents in the plasma membrane of VMH neurons in *Kir6.2*$^{+/+}$, we dialyzed the cells with ATP-free pipette solution (Fig. 3a). In all three types of *Kir6.2*$^{+/+}$ VMH neurons ($n = 20$), the dialysis induced cessation of spontaneous activity and membrane hyperpolarization (control, –40.1 ± 1.3 mV; ATP washout, –61.5 ± 1.9 mV; $n = 20$, $p < 0.001$; Fig. 3a, left). This effect was blocked by the sulfonylurea tolbutamide. By contrast, ATP washout had no effect on the electrical properties of any type of VMH neurons in *Kir6.2*$^{-/-}$ mice (0/22; control, –34.1 ± 1.2 mV; ATP washout, –35.8 ± 1.6 mV, $n = 22$; Fig. 3a, right), suggesting the absence of the K_{ATP} channels in VMH neurons in *Kir6.2*$^{-/-}$. We next recorded K_{ATP} channel currents using the whole-cell voltage-clamp configuration (Fig. 3b). In all three types of VMH neurons in *Kir6.2*$^{+/+}$, dialysis with ATP-free pipette solution activated time- and voltage-independent membrane currents (48.8 ± 6.9 pA at –50 mV, $n = 20$; 11 type A, 3 type B, and 6 type C neurons; Fig. 3b, upper left) that reversed close to E_K (–93.1 ± 2.0 mV, $n = 10$; Fig. 3b, middle left). The currents were completely blocked by tolbutamide, indicating that they flowed through K_{ATP} channels. In contrast to *Kir6.2*$^{+/+}$ neurons, dialysis with ATP-free solution did not activate currents in VMH neurons of *Kir6.2*$^{-/-}$ mice (–0.5 ± 1.6 pA, $n = 20$; Fig. 3b, upper and middle right), demonstrating the complete absence of K_{ATP} channels in the plasma membrane. The IC_{50} (8.2 μM) for tolbutamide block is consistent with the K_{ATP} channels containing SUR1 (ref. 35; Fig. 3b, bottom right panel).

The K_{ATP} subunits in VMH neurons

The electrophysiological data on *Kir6.2*$^{-/-}$ clearly showed Kir6.2 to be the pore-forming subunit of the plasma membrane K_{ATP} channels in VMH neurons. In addition, the sensitivity of the K_{ATP} channels to tolbutamide strongly suggests that SUR1 is the sulfonylurea receptor subunit. To define the molecular composition of the K_{ATP} channel directly, the expression of the K_{ATP} channel subunit mRNA was determined in all three types of

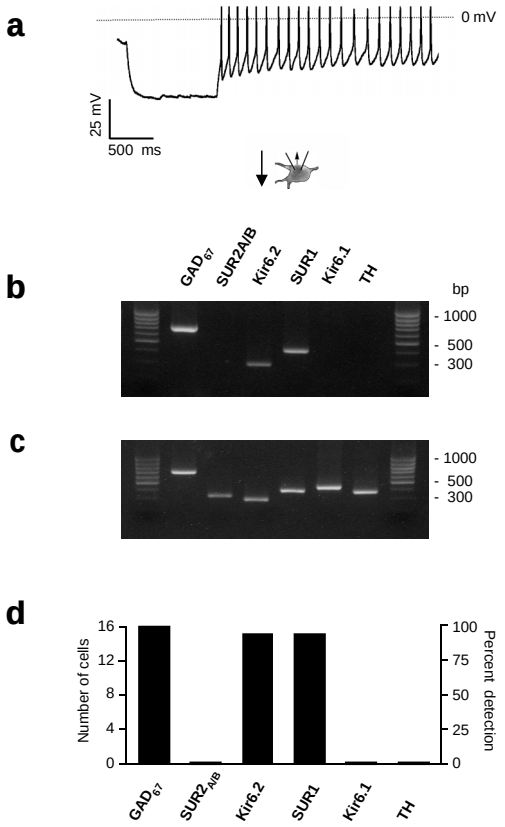

Fig. 4. Single-cell RT-PCR of K_{ATP} channel subunits in VMH neurons in *Kir6.2*$^{+/+}$. (**a**) Whole-cell current-clamp recording of a VMH neuron. Fast repolarization after injection of –50 pA hyperpolarizing current identified this neuron as type A (compare with **Fig. 2a**). The cytoplasm of this neuron was harvested for single-cell RT-PCR analysis. (**b**) Agarose gel analysis of single-cell RT-PCR. The same neuron as shown in (**a**) was analyzed. PCR products corresponding to GAD_{67}, Kir6.2 and SUR1 were detected. No Kir6.1, SUR2A or SUR2B was detected in this cell. (**c**) Positive control for RT-PCR. Mouse hypothalamic cDNA was used as the template for the positive control for K_{ATP} channel subunits, GAD and TH. The predicted sizes (bp) of the amplified PCR products were as follows: GAD_{67}, 701 bp; SUR2B, 337 bp; Kir6.2, 297 bp; SUR1, 400 bp; Kir6.1, 447 bp; TH, 376. (**d**) Summary of single-cell RT-PCR of VMH neurons (total 16 neurons; 8 type A, 2 type B, and 6 type C neurons).

VMH neurons in *Kir6.2*$^{+/+}$, using the single-cell reverse transcription (RT)-multiplex polymerase chain reaction (PCR) technique[20,37]. The electrophysiological phenotype of the VMH neuron was determined before harvesting the cytoplasm for expression profiling (Fig. 4a). We analyzed 16 neurons (8 type A, 2 type B, and 6 type C neurons; Fig. 4b–d), all of which expressed glutamate decarboxylase (GAD_{67}) (16/16) but not tyrosine hydroxylase (TH) mRNA, indicating the GABAergic phenotype. Both Kir6.2 and SUR1 were detected in all three types of VMH neurons examined (15/16). In contrast, no Kir6.1, SUR2A or SUR2B was detected in single VMH neurons.

Food intake in response to 2DG, Leptin and NPY

In addition to stimulating glucagon secretion, 2DG also increases food intake in normal mice, presumably by inhibiting the activity of GR neurons in the hypothalamus[37]. *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ mice were injected with 2DG, and the food intake was monitored for 3 h after injection. The increment in food intake of *Kir6.2*$^{-/-}$ mice (0.095 ± 0.037 g/3 h, n = 28) was significantly less than that of *Kir6.2*$^{+/+}$ mice (0.279 ± 0.039 g/3 h, n = 27, $p < 0.002$; Fig. 5a), indicating a functional role for hypothalamic K_{ATP} channels in the control of food intake. Leptin, an adipocyte hormone that reduces food intake[38], inhibits hypothalamic neurons by activating K_{ATP} channels[39]. Leptin inhibited food intake in *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ to a similar degree (Fig. 5b), demonstrating that the effect of leptin on food intake is independent of Kir6.2-containing K_{ATP} channels. Neuropeptide Y (NPY), which increases food intake[40], was also similarly effective in *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ (Fig. 5c).

Discussion

The hypothalamus regulates the secretion of counter-regulatory hormones such as glucagon and catecholamines through the autonomic nervous system, and thus is critical in glucose homeostasis[30,41]. A subset of hypothalamic neurons clustered in the VMH respond to elevated extracellular glucose levels[42] by increasing their firing rates. Our findings in the present study demonstrate that K_{ATP} channels comprising Kir6.2 and SUR1 have a key role in this glucose-sensing process.

Kir6.2$^{-/-}$ mice exhibited impaired glucagon secretion during systemic hypoglycemia, despite the fact that glucagon secretion in response to a low concentration of glucose from isolated pancreatic islets was normal. In addition, intracerebroventricular administration of 2DG did not elicit glucagon secretion in *Kir6.2*$^{-/-}$. These *in vivo* findings indicate that the primary defect in glucagon secretion in *Kir6.2*$^{-/-}$ is not in the pancreatic α-cell itself but rather in its hypothalamic regulation. In support of these findings, *in vitro* brain slice experiments revealed the complete loss of glucose sensing in VMH associated with lack of functional K_{ATP} channels in the plasma membrane of *Kir6.2*$^{-/-}$ VMH neurons. These results demonstrate that Kir6.2 forms the pore

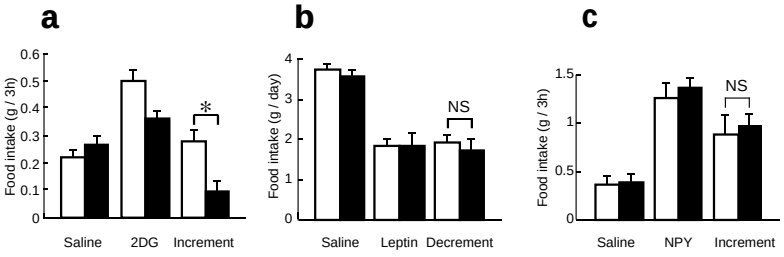

Fig. 5. The effects of 2DG, leptin and NPY on food intake in *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ mice. (**a**) Effect of 2DG on food intake. Food intake for 3 h after saline injection (x) was measured. Three days later, food intake for 3 h after 2DG administration (y) was measured in the same mice. The increment in food intake in the same mice was calculated by subtracting x from y. The increments were as follows: *Kir6.2*$^{+/+}$, 0.279 ± 0.039 g/3 h, n = 27; *Kir6.2*$^{-/-}$, 0.095 ± 0.037 g/3 h, n = 28, $^*p < 0.002$. (**b**) Effect of leptin on food intake. The decrement in food intake due to leptin was calculated similarly to (**a**). The cumulative food intake was measured over 24 h. (**c**) Effect of NPY on food intake. The increment in food intake due to NPY was calculated similarly to (**a**). Open columns and filled columns represent *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$, respectively. The data were obtained from 7–30 samples in (**a**–**c**). The values are means ± s.e.m. NS, not significant.

of the K_{ATP} channel in hypothalamic GR neurons, and that this channel is an essential part of the neuronal glucose sensor that couples alterations in extracellular glucose levels to changes in neuronal excitability. We also provide evidence that the K_{ATP} channels in the VMH regulate peripheral glucagon secretion in response to neuroglycopenia. Mutation of *Kir6.2* or *SUR1* causes familial persistent hyperinsulinemic hypoglycemia of infancy (PHHI)[16]. In addition to the unregulated insulin secretion, the present findings suggest that impaired glucagon secretion due to K_{ATP} channel dysfunction in the hypothalamus might also contribute to the prolonged hypoglycemia in these patients.

The molecular composition of K_{ATP} channels in GR VMH neurons is controversial[21,43–45]. Our results show that all three types of VMH neurons express both Kir6.2 and SUR1, but that only a subset of these neurons are glucose responsive. Thus, coexpression of Kir6.2 and SUR1 is necessary but not sufficient for hypothalamic glucose sensing. It seems likely that the different glucose sensitivities of VMH neurons result from differential expression of genes involved in glucose metabolism. Analogously to the pancreatic β-cell, these might include the low-affinity glucose transporter GLUT2[46] and the glucose-phosphorylating enzyme glucokinase[47]. Consistent with this possibility, the expression of both GLUT2 and glucokinase isoform have been reported in VMH neurons[34,43,48].

Our results from the 2DG-induced food intake experiments strongly suggest that the K_{ATP} channels in the hypothalamus are also involved in the control of appetite. Studies have shown that food intake is controlled by complex hormonal and neuronal signaling pathways[49]. The adipocyte hormone leptin, one of the key signals of satiety, has been reported to activate hypothalamic K_{ATP} channels acutely *in vitro*[39]. However, it is not known if K_{ATP} channels are important in mediating the anorexic effect of leptin. We show here that the effects of both leptin and the orexic peptide NPY on food intake are independent of the K_{ATP} channels in VMH neurons. Thus, the mechanism of K_{ATP} channel-mediated regulation of food intake is distinct from that of leptin and NPY. Because leptin and NPY affect the long-term regulation of body weight[49], the K_{ATP} channels in VMH neurons might be involved in short-term regulation of food intake, by coupling glucose levels to appetite.

The present study shows that genetic disruption of *Kir6.2* deprives the VMH neurons of both K_{ATP} channel activity and glucose responsiveness. In addition, activation of the K_{ATP} channels in the GR neurons is critical for stimulation of both glucagon secretion and food intake when brain glucose levels fall. On the other hand, inhibition of the pancreatic β-cell K_{ATP} channels is essential for glucose-dependent insulin secretion when blood glucose levels rise[22]. Thus, hypothalamic and β-cell K_{ATP} channels not only share the same molecular composition but also act in concert as central and peripheral glucose sensors to coordinate the maintenance of glucose homeostasis.

Methods

Mice. Mice lacking *Kir6.2* were generated as described[22]. The mutant mice and wild-type mice were backcrossed for five generations to a C57Bl/6 background. All the *in vivo* experiments began at around 8:00–10:00 a.m. All animal procedures were approved by the Chiba University and Oxford University Animal Care Committees.

Glucagon and catecholamine secretion *in vivo*. Male mice 12–20 weeks old were deprived of food for 16 h before the experiments. Blood samples were drawn from the orbital sinus. Glucagon secretion induced by insulin-induced hypoglycemia was examined before and 60 min after the intraperitoneal injection of human insulin (1 U/kg). Glucagon secretion induced by 2DG was examined before and 15 min after intracerebroventricular injection of 2DG (1 mg per body) to conscious mice that had been stereotaxically implanted with a stainless cannula in the third intracerebroventricle five to seven days before the experiment. Catecholamine was measured 60 min after intraperitoneal injection of human insulin (1 U/kg) or saline.

Glucagon secretion *in vitro*. Glucagon secretion from isolated pancreatic islets was measured using a previously designed method[50], slightly modified. Briefly, pancreatic islets were isolated by the collagenase method and were handpicked under a stereomicroscope at room temperature. The freshly isolated islets were preincubated at 37°C for 60 min in Krebs–Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mM HEPES, 0.1% bovine serum albumin and 7 mM glucose. The islets (10 in each tube) were incubated for 60 min in 500 μl of the Krebs–Ringer bicarbonate buffer containing 7 mM glucose plus 50 μM carbachol, 16.7 mM glucose or 1 mM glucose in the presence of 500 KIU/ml of aprotinin. Immediately after incubation, aliquots (350 μl) of the medium were removed for assay of glucagon.

Measurements of blood glucose, glucagon and catecholamine. Blood glucose levels were measured as described[22]. The glucagon levels in plasma or perfusate were measured by RIA kit from LINCO (Linco, Missouri). The plasma catecholamine levels were measured by HPLC with fluorescence detection (Tosoh, Tokyo, Japan).

Electrophysiology. Coronal brain slices (250 μm) containing the VMH were prepared from 13–16-day-old *Kir6.2*$^{-/-}$ and *Kir6.2*$^{+/+}$ mice, and electrophysiological brain slice patch-clamp recordings were done using DIC-IR optics, as previously described[20]. Briefly, after at least 30 min recovery, midbrain slices were transferred for patch-clamp recordings into a chamber continuously perfused with artificial cerebrospinal fluid (ACSF) at 2–4 ml/min with either high (25 mM glucose) or low glucose (2.5 mM glucose plus 22.5 mM sucrose). ACSF was bubbled with a mixture of 95% O_2 and 5% CO_2 at 37°C. For whole-cell recordings, patch pipettes (1–2.5 MΩ) were pulled from thin-walled borosilicate glass (Clark, Reading, UK) and filled with internal solution containing 120 mM K-gluconate, 20 mM KCl, 10 mM HEPES, 10 mM EGTA and 2 mM $MgCl_2$, pH 7.3 (290–300 mOsm). For cell-attached recordings, patch pipettes (5–6 MΩ) pulled from borosilicate glass (Clark) were filled with an external solution containing 150 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 2 mM $MgCl_2$ and 2 mM $CaCl_2$, pH 7.4 (290–300 mOsm). The Pulse/Pulsefit (Heka, Lambrecht, Germany) program was used for data acquisition and Igor (Wavemetrics, Lake Oswego, Oregon) for analysis. Recordings were digitized at 2–5 kHz and filtered with a low-pass filter Bessel characteristic of 1 kHz cutoff frequency.

Multiplex and nested single-cell RT-PCR. Single-cell RT-multiplex PCR experiments, including primer sequences and PCR protocols, were done as previously described[20]. Briefly, after reverse transcription, the cDNAs for the K_{ATP} channel subunits (Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B), tyrosine hydroxylase (TH), and the 67 kD form of glutamate decarboxylase (GAD_{67}) were simultaneously amplified in a first multiplex PCR, followed by a second amplification in individual nested PCR reactions. Aliquots (15 μl) of PCR products were separated and visualized in an ethidium-bromide-stained agarose gel (2%) by electrophoresis (predicted sizes of the PCR-generated fragments, GAD67, 702 bp; SUR2A, 513 bp; SUR2B, 337 bp; Kir6.2, 297 bp; SUR1, 400 bp; Kir6.1, 447 bp; TH, 377 bp). All individual PCR products were verified by direct sequencing. Mouse hypothalamic cDNA (< 10 fmol) was used as template for positive controls.

Measurements of food intake. Mice eating *ad libitum* were injected with 2DG (500 mg/kg), mouse leptin (4 μg) or rat NPY (2 μg) at 9:00 a.m. As a control, vehicle (saline) was injected. 2DG was administered intraperitoneally; leptin and NPY were administered intracerebroventricularly. Accumulative food intake was measured for 3 h (2DG and NPY) or 24 h (leptin) after injection. The food used was normal mouse chow, CE-2 (Japan Clea, Tokyo, Japan).

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