Hypothalamic POMC-deficiency improves glucose tolerance despite insulin resistance by increasing glycosuria

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Abstract

Hypothalamic proopiomelanocortin (POMC) is essential for the physiological regulation of energy balance; however its role in glucose homeostasis remains less clear. Here, we show that hypothalamic arcuate nucleus (Arc) POMC-deficient mice, which develop severe obesity and insulin resistance, unexpectedly exhibit improved glucose tolerance and remain protected from hyperglycemia. To explain these paradoxical phenotypes, we hypothesized that an insulin-independent pathway is responsible for the enhanced glucose tolerance. Indeed, the mutant mice demonstrated increased glucose effectiveness and exaggerated glycosuria relative to their wildtype controls at comparable blood glucose concentrations. Central administration of the melanocortin receptor agonist melanotan II in mutant mice reversed both their alterations in glucose tolerance and glycosuria, while conversely, administration of the antagonist Agrp to wildtype mice enhanced their glucose tolerance. The glycosuria of ArcPOMC-deficient mice was due to decreased levels of renal glucose transporter 2 (rGLUT2), but not sodium/glucose cotransporter 2 (SGLT2), and was associated with reduced renal catecholamine content. Epinephrine treatment abolished the genotype-differences in glucose tolerance and rGLUT2 levels, suggesting that reduced renal sympathetic nervous system (SNS) activity is the underlying mechanism for the observed glycosuria and improved glucose tolerance in ArcPOMC-deficient mice. Therefore, the ArcPOMC-SNS-rGLUT2 axis is potentially an insulin-independent therapeutic target to control diabetes.
Introduction

Hypothalamic neurons integrate signals from metabolites like pyruvate (1) as well as hormones such as insulin (2; 3), leptin (4), and GLP-1 (5) in the CNS control of glucose homeostasis. Disruption of hypothalamic leptin and insulin signaling leads to insulin resistance (6), indicating a physiological role of the hypothalamus in blood glucose regulation. Moreover, leptin receptors in hypothalamic POMC neurons regulate glycemia independently of changes in food intake (7). Interestingly, POMC neurons directly sense glucose and this property is impaired in obesity (8; 9). Recently, Williams et al. (10) and Smith et al. (11) identified the contribution of X-box binding protein 1 and S6K1, respectively, in POMC neurons in the regulation of insulin sensitivity and/or hepatic glucose production. Overall, these studies and others clearly validate POMC neurons as a potential therapeutic target to control hyperglycemia.

The melanocortin (MC) system, which originates in the POMC neurons, regulates energy balance through MC-3 and 4 receptors. MC4 receptor (MC4R) mutations cause hyperphagia, increased bodyweight, and insulin resistance in mice and humans (12-14). In contrast, MC3R-deficient mice exhibit a mild obesity syndrome associated with defects in nutrient partitioning despite normal food intake and energy expenditure (15; 16). Mice lacking both MC4R and MC3R are significantly heavier than MC4R knockout mice suggesting that these receptors have non-redundant roles in the regulation of energy balance (15). Notably, MC4R deficient mice do not exhibit fasting hyperglycemia or impaired glucose tolerance - the hallmark symptoms of diabetes - despite insulin resistance and obesity (12; 13). Clinical data (14) also support this observation suggesting that an insulin-independent pathway could be responsible for maintaining normoglycemia in subjects lacking melanocortin signaling.
The POMC polypeptide is synthesized mainly in the pituitary gland and the hypothalamic arcuate nucleus (Arc). POMC negatively regulates energy homeostasis (17-19) and consequently ArcPOMC-deficiency causes obesity due to hyperphagia and decreased energy expenditure (19). While the physiological significance of central POMC in body weight (19) and blood pressure regulation (20) is well established, its role in maintaining glucose homeostasis is less defined. Central Pomc expression is secondarily reduced in leptin-deficient obese, ob/ob, and leptin-receptor deficient diabetic, db/db, mice (21) suggesting the involvement of POMC in leptin-associated obesity and diabetes. It has been demonstrated that restoration of Pomc expression in ob/ob (22) and obese Pomc knockout mice (19) improves glucose tolerance and/or fasting glycemia, however these studies did not take into account the secondary effects of obesity as the experiments were carried out in mice that were obese and/or concurrently hyperglycemic. Moreover, an abnormal counterregulatory response to hypoglycemia has been reported in global POMC-null mice (23), which lack peripheral as well as central melanocortin signaling; but the specific function of hypothalamic POMC in glucose homeostasis remains to be established. In this study, we utilized ArcPOMC-deficient mice (19) generated by our laboratory to further determine the function of hypothalamic POMC in the regulation of glycemia. We measured glucose and insulin tolerance in groups of mutant mice that were either obese or weight-matched to wildtype controls by food-restriction to exclude secondary effects of obesity.
Research Design and Methods

Study approval: All procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan and followed the Public Health Service guidelines for the humane care and use of experimental animals.

Animal care: Mice were housed in ventilated cages under controlled temperature (~23°C) and photoperiod (12-hour light/ 12-hour dark cycle, lights on from 6 a.m. to 6 p.m.), with tap water and laboratory chow (LabDiet, 5L0D) containing 28.5 kcal% protein, 13.5 kcal% fat, and 58.0 kcal% carbohydrate either available ad libitum or restricted according to the approved experimental protocol. Weight-matched ArcPOMC-deficient mice were fed 75-80% of the daily total food consumed by their WT littermates starting immediately after weaning to prevent their development of obesity. Wildtype and mutant mice were housed individually for experiments involving weight-matching.

Generation and breeding of mice: ArcPOMC-deficient mice were generated and bred as described in detail previously (24). These mice have an identical phenotype to those described by Bumaschny et al., 2012 (19) due to the transcriptional blocking effects of a neoR cassette inserted in the neural enhancer region of the Pomc gene. However, they also have deletions of both nPE1 and nPE2 as shown in Figure S3 of the previous report (24). These mice have no detectable Pomc expression in the hypothalamic arcuate nucleus but intact expression in the nucleus tractus solitarius and the pituitary gland. The mutant strain was backcrossed for at least 5 generations on to the C57BL/6J genetic background and these incipient congenic mice were used throughout the study except for the results presented in Figure 2. Those experiments used mutant and wildtype siblings derived from the founding chimera after at least 5 generations of backcrossing to the 129S6/SvEvTac genetic background.
Oral glucose- and intraperitoneal insulin- tolerance tests: Mice were fasted on separate occasions for 6 hours (8:00 am to 2:00 pm) before subjecting them to the tests. For oral glucose tolerance tests (OGTT), glucose (fixed dose of 60 mg per mouse, Sigma G5767) was delivered into the stomach by a gavage needle (18-gauge, FNS-18-2; Kent Scientific Corporation), and blood was sampled at 0, 15, 30, 60, and 120 min for glucose measurements by an AlphaTRAK 2 glucometer. A fixed dose of glucose was given, rather than a dose adjusted by body weight, to eliminate the confounding effects of obesity. This method is recommended and has been validated for evaluating glucose tolerance in mice (25; 26). Moreover, glucose tolerance in humans is also assessed based on a fixed dose (75 g) of glucose administration. For experiments involving epinephrine (Figure 7D), the OGTT was carried out 60 minutes after epinephrine (0.3 mg/kg lean mass, i.p.) or saline injection.

For insulin tolerance tests, insulin (0.5 U/kg lean mass, i.p., HumulinR, Lilly) was administered and blood was sampled at 0, 15, 30, 60, and 120 min for glucose measurements as described for OGTT. The total area under the curve (AUC) was calculated using the Trapezoidal rule.

Frequently sampled intravenous glucose tolerance test (FSIVGTT): A FSIVGTT (27) was performed in 6 h fasted ArcPOMC-deficient mice or their littermate controls four or five days after carotid arterial and jugular venous catheterization. The catheterizations were performed as described previously (28). Blood sampling was performed via the arterial catheter in unrestrained, conscious animals. A baseline fasted blood sample was obtained followed immediately by the i.v. administration of a fixed dose of 60 mg of glucose per mouse over a period of 20 seconds at t = 0 min. Blood was then sampled for measurement of glucose and insulin at t = 1, 2, 4, 7, 10, 15, 20, 30, and 60 min. The acute insulin response to hyperglycemia
was based on the area under the curve of insulin levels between 0 and 10 min. The data were analyzed by the minimal model method to calculate glucose effectiveness as described previously (27; 29) using MLAB (Civilized Software Inc.).

**Urine glucose levels:** Urine glucose levels were measured using a spot urine test (Bayer Diastix, 2803) during OGTTs at the different time points indicated in the Supplementary Tables. For 24 hr urine collections, mice were housed individually in metabolic cages (Techniplast) and acclimatized to the cages for a week before undergoing experiments. Urine was collected for 24 hr before and after OGTT and the glucose concentration was quantified using a Siemens ADVIA 1800 chemistry analyzer (Siemens Healthcare, Tarrytown, NY) by a coupled enzymatic procedure with hexokinase and glucose 6-phosphate dehydrogenase (G6PDH).

**10% glucose challenge test:** Mice were provided 10% glucose in their drinking water for 24 hr. Urine was collected and its glucose concentration was quantified as described above.

**Liver glycogen content and pyruvate tolerance test:** Hepatic glycogen levels were measured using a kit from Sigma (MAL016). For pyruvate tolerance tests (PTT), sodium pyruvate (fixed dose of 60 mg per mouse, Sigma P5280) was injected i.p. into mice after a 6 hr fast and blood glucose was measured at 0, 15, 30, 60, and 120 min post injection.

**Renal epinephrine/norepinephrine and plasma insulin measurements:** Mice were euthanized at 9:00 am and the kidneys were collected under baseline (no treatment) conditions. Whole kidneys were homogenized in 0.01 N HCl and the concentration of catecholamines was measured with an ELISA as described by the manufacturer (LDN®, BA E-5400). For plasma insulin measurements, mice were fasted for 6 h (8:00 am to 2 pm) before collecting their tail-vein blood using Fisherbrand™ microhematocrit capillary tubes (22-362-566). The blood was
centrifuged at 4°C for 20 min. at 2,000 x g and the plasma was assayed using an ultra-sensitive mouse insulin ELISA kit (Crystal Chem, 90080).

**Intracerebroventricular (icv) melanotan II (MTII) and Agrp treatments:** Mice were anesthetized with 2-4% isoflurane. Twenty-six–gauge stainless steel guide cannulae cut 2.5 mm below the pedestal (Plastics One, Roanoke, VA) were implanted stereotaxically into the left lateral ventricle (A-P -0.5 mm, M-L -1.0 mm, D-V -2.0 relative to bregma), secured to the skull using screws (Small Parts) and dental cement, and occluded with stainless steel dummy obturators. Mice were then housed individually for 7–10 days of recovery. MTII (0.5 µg, Sigma M8693) or Agrp (1 µg, Phoenix Pharma 003-57) were injected icv t.i.d. in a volume of 5 µl over 5 min using a 33-gauge stainless steel injection cannula extending 0.5 mm below the guide cannula and connected to a 25 µl Hamilton syringe with polyethylene tubing. The last dose was administered 30 min prior to OGTT.

**Western blot:** Mouse kidneys were harvested 60 min post oral glucose administration (fixed dose of 60 mg) for the determination of differences in GLUT2 or SGLT2 levels between the genotypes. For experiments involving saline or epinephrine treatment, the kidneys were removed 60 minutes after the treatment and immediately frozen on dry ice. The renal cortical tissue was separated from the medullary portion under magnification with a dissecting microscope using a razor blade and suspended in 10 times volume of ice-cold RIPA buffer (Pierce, 89900) containing protease inhibitors (Pierce, 87786). The tissue was minced with scissors and further homogenized using an Omni tissue homogenizer. The homogenate was centrifuged for 20 min at 12,000 rpm at 4°C in a microcentrifuge and the pellet was discarded. The supernatant was analyzed for protein concentration by a BCA method (Pierce, Thermo Scientific, 23227). The lysate containing 50 mg protein was mixed with an equal volume of Laemmli buffer (Biorad,
161-0737) before heating at 95°C for 10 min. SDS-PAGE was performed on TGX 4–20% gradient gels (Biorad, 456-1094) followed by a semi-dry transfer of the proteins on to PVDF membranes (Millipore). The membranes were blocked in 5% non-fat dry milk solution in tris-buffered saline tween 20 (TBST) for 1 h before incubating them with primary antibodies overnight at 4°C. Rabbit polyclonal anti-GLUT2 serum (Rockland Immunochemicals, 100-401-GN3 or Millipore, 07-1402), anti-GLUT2 affinity purified (Rockland Immunochemicals, 600-401-GN3), and goat polyclonal anti-SGLT2 (Santa Cruz, sc-47402) antibodies were diluted 1:2,000 and 1:1,000, respectively, in TBST containing 5% powdered milk. After incubation, membranes were washed three times (15 min/wash) using TBST. Following the washes, membranes were incubated with the secondary antibodies, anti-rabbit (GE Healthcare, NA934) or anti-goat IgGs (Santa Cruz, sc-2768) coupled to horseradish peroxidase. Vinculin expression was evaluated on the same membranes to confirm equal sample-loading using Abcam, ab73412 antibody. Luminescence was generated with the Amersham ECL advance Western blotting detection kit (GE Healthcare) and recorded on an Imaging System. The correct band size, 50 KDa, for GLUT2 was confirmed using a HEK293 cell lysate transfected with a GLUT2 expression vector (Santa Cruz sc-120518) as a positive control. We also confirmed our data using anti-GLUT2 serum provided by Dr. Bernard Thorens’ laboratory. GLUT2 antibodies used in this study identified non-specific bands at 75 and 120 KDa in kidney samples.

**Statistics:** All data are presented as mean ± s.e.m. and were analyzed by Student’s unpaired two-tailed or Welch’s t test, or two-way ANOVA followed by Bonferroni’s test when appropriate using GraphPad Prism 6. Some data, as identified in the figures, were log-transformed to convert them into a normal distribution. P values less than 0.05 were considered significant.
Results

Obese and Weight-matched ArcPOMC-deficient mice exhibit improved glucose tolerance compared to wild-type littermates

We measured fasting plasma insulin and determined whole body insulin sensitivity in obese ArcPOMC-deficient mice (Figure 1A). The ArcPOMC-deficient mice exhibited hyperinsulinemia and reduced insulin sensitivity compared to their WT littermates (Figure 1B,C,D). Additionally, the mutant mice had a higher HOMA-IR index (Male, 169.1 ±35 vs. 9.0 ±2; Female, 165.3 ±35 vs. 8.5 ±2.3 mM * mU/L, P<0.001), which is highly correlated with reduced insulin sensitivity (25), than the control group. These results are consistent with previous reports suggesting a role for central melanocortins in the regulation of insulin action (12-14). Next, we performed oral glucose tolerance tests (OGTT), the most reliable method to assess glucose tolerance in mice (25). Paradoxically, we observed improved - rather than impaired - glucose tolerance in obese ArcPOMC-deficient mice (Figure 1E,F) despite their insulin resistance. Furthermore, 52-week old ArcPOMC-deficient mice did not exhibit fasting hyperglycemia or impaired glucose tolerance (data not shown) despite obesity, hyperphagia, and insulin resistance. The phenotype of improved glucose tolerance in the presence of reduced insulin sensitivity was also confirmed in obese 129S6 background ArcPOMC-deficient mice (Figure 2).

To eliminate a possible confounding or secondary effect of obesity that might have affected insulin and glucose metabolism in ArcPOMC-deficient mice, we also determined insulin sensitivity and performed OGTTs with ArcPOMC-deficient mice that were weight-matched to wildtype (WT) littermates by calorie restriction (Figure 3A). Fasting plasma insulin was significantly higher and insulin sensitivity was lower in weight-matched ArcPOMC-deficient
mice (Figure 3B,C,D) compared to their WT littermates. Moreover, consistent with the obese ArcPOMC-deficient mice, the weight-matched mutant mice also had a higher HOMA-IR index than the control groups (Male, 13.4 ±1.8 vs. 7.3 ±1.7; Female, 12.0 ±1.2 vs. 6.2 ±1.1 mM * mU/L, respectively, P<0.05). These data suggest that insulin resistance in ArcPOMC-deficient mice occurs independently of total body weight and is a direct outcome of ArcPOMC-deficiency, similar to what has been observed in MC4R knockout mice that exhibit reduced insulin sensitivity even before the onset of obesity or hyperphagia (13). Surprisingly, despite their insulin resistance, the weight-matched ArcPOMC-deficient mice also showed improved glucose tolerance (Figure 3E,F), indicating that the paradoxical phenotype is independent of changes in body weight and is a direct consequence of ArcPOMC-deficiency. Moreover, 24-wk old weight-matched ArcPOMC-deficient mice also exhibited improved glucose tolerance (data not shown) suggesting that the phenotype was independent of changes in age. It should be noted, however, that weight-matched ArcPOMC-deficient mice have a mild persistent elevation in their percentage of body fat composition measured by NMR (data not shown).

We performed a frequently sampled intravenous glucose tolerance test (FSIVGTT) (27; 30) to determine if the observed improvement of glucose tolerance in ArcPOMC-deficient mice depended on the route of administration. FSIVGTT data also revealed an improvement in glucose tolerance (Figure 4A,B) in ad lib fed ArcPOMC-deficient mice (8-wk old, 28 ±0.6 g body weight) compared to the control WT group (8-wk old, 22 ±0.3 g body weight). The initial peak insulin concentration after glucose challenge was 3 fold higher than the baseline in WT mice, however in mutant mice the peak was only 1.3 fold higher than their already elevated fasting insulin levels (Figure 4C). Consistent with results of FSIVGTT in ob/ob mice (27; 30), ArcPOMC-deficient mice exhibited hyperinsulinemia (Figure 4C) throughout the test, except for
the initial peak, and hence an elevated acute insulin response to glucose integrated over the first ten minutes (674.3 ±69 vs. 469.3 ±52 mU/ml * min, \( P < 0.05 \)), supporting the HOMA-IR data and confirming insulin resistance in the mutant mice as mentioned above. Interestingly, glucose effectiveness, defined as insulin-independent glucose disposal (30), was increased in the ArcPOMC-deficient mice (Figure 4D) compared to their WT littermates suggesting that an insulin-independent mechanism mediates the improvement in glucose tolerance. These data confirm that the ArcPOMC-deficient mice exhibit improved glucose tolerance despite insulin resistance.

**ArcPOMC-deficient mice show elevated glycosuria but normal hepatic glycogen levels and gluconeogenesis**

To explain the paradox of improved glucose tolerance in the presence of insulin resistance, we hypothesized that ArcPOMC-deficient mice would exhibit exaggerated glycosuria – an insulin-independent mechanism of glucose disposal that is currently utilized as a therapy to control diabetes (31) – compared to WT littermates. To test this hypothesis, we analyzed urine glucose levels under different glucose-challenged conditions. Weight-matched ArcPOMC-deficient mice showed elevated glycosuria at all time-points during OGTT compared to WT mice (Supplementary Table 1A), even though their corresponding blood glucose levels were lower than WT mice (Figure 3 E,F). Moreover, urine from the mutant mice collected over 24 hr in metabolic cages post OGTT also showed a higher glucose concentration than that of the control group (Figure 5A). To further confirm the reduced threshold for renal glucose reabsorption in the mutant mice, we provided 10% glucose in drinking water for 24 hr and collected urine in metabolic cages. We found a profound increase in urinary glucose excretion by the mutant compared to WT mice (Figure 5B), further validating our hypothesis of suppressed
renal glucose reabsorption in ArcPOMC-deficient mice. Obese ad libitum fed ArcPOMC-deficient mice showed elevated glycosuria (93.5 ±14 vs. 48 ±5 mg/dl, P<0.05) even without glucose challenge, unlike the calorie restricted and weight-matched mice. Additionally, the obese mice, but not the calorie restricted mice, exhibited albuminuria (10.4 ±2.6 vs. 1.1 ±0.5 µg/day, P < 0.0001) compared to the WT group, suggesting secondary effects of obesity on kidney pathology. Interestingly, both obese and weight-matched mutant mice had elevated natriuresis (0.5 ± 0.02 and 0.4 ± 0.03, respectively, vs. 0.3 ± 0.01 mmol/day, P < 0.05). Decreased renal sympathetic nervous system activity in the mutant mice can possibly explain the observed natriuresis.

In addition to glycosuria, hepatic glycogen turn-over and gluconeogenesis contribute to glucose homeostasis. Moreover, hypothalamic α-MSH regulates hepatic gluconeogenesis (32). Hence, we measured liver glycogen levels and evaluated gluconeogenesis by a pyruvate tolerance test to determine if changes in these parameters are responsible for protecting ArcPOMC-deficient mice against hyperglycemia in the presence of insulin resistance. We found no differences in either hepatic glycogen levels (baseline and post glucose challenge, Figure 5C) or gluconeogenesis (pyruvate tolerance test, Figure 5D,E) between ArcPOMC-deficient mice and their WT littermates. Therefore, a reduced threshold for glucose reabsorption appears to be the major mechanism that is responsible for the enhanced glucose tolerance phenotype in ArcPOMC-deficient mice.
Pharmacological experiments with MTII or Agrp support results from the ArcPOMC-deficient genetic mouse model

We treated ArcPOMC-deficient mice with MT II (0.5 µg/5 µl PBS, icv, t.i.d.), a non-selective MC receptor agonist, to validate if the phenotype of improved glucose tolerance and glycosuria is attributable to decreased MC signaling in the POMC-deficient mice. Remarkably, MTII treatment reversed the phenotype and resulted in normal, rather than enhanced, glucose tolerance (Figure 6A) concomitantly with the absence of exaggerated glycosuria (Supplementary Table 2A) compared to the same set of mice after saline injections. Consistent with our previous study (33), MTII decreased food intake and body weight dramatically in the mutant mice (Figure 6B,C). A WT group treated with MTII was not included in the study because our main goal was to ascertain if MTII can reverse the phenotype of ArcPOMC-deficient mice.

To pharmacologically simulate the reduced central MC signaling of ArcPOMC-deficient mice, we treated WT mice with Agrp (1 µg/5 µl PBS, icv, t.i.d.), a MC3/4 receptor antagonist. Consistent with the results from the mutant mice, Agrp treated WT mice had improved glucose tolerance (Figure 6A) and elevated glycosuria (Supplementary Table 2B) at 15 and 60 min interval during OGTT relative to the same set of mice following saline administration. However, unlike the mutant mice, glycosuria was absent at the 120 min time point during OGTTs in Agrp treated WT mice. Expectedly, Agrp increased body weight and food intake in WT mice (Figure 6B,C).

ArcPOMC-deficient mice have reduced renal GLUT2 but not SGLT2 levels

To determine the molecular mechanisms underlying exaggerated glycosuria in ArcPOMC-deficient mice, we evaluated the expression of the major renal (r) proximal tubule
glucose transporters, GLUT2 and SGLT2, 60 minutes after oral glucose administration (fixed dose of 60 mg per mouse). We observed a significant decrease in rGLUT2, but not rSGLT2, protein levels (Figure 7A,B) in the mutant mice suggesting that suppression of rGLUT2 mediates glycosuria in ArcPOMC-deficient mice.

**Epinephrine abolishes the genotype differences between WT and ArcPOMC-deficient mice**

Because of the enriched sympathetic innervation of kidneys (34) and the demonstrated direct relationship between central POMC signaling and sympathetic outflow (35-37), we questioned whether reduced sympathetic tone contributes to the observed down regulation of rGLUT2 in ArcPOMC-deficient mice. Indeed, we found that kidney norepinephrine (NE) and epinephrine (E) levels were decreased by 50% in the mutant mice compared to controls (Figure 8A), consistent with a previous clinical report (38) that demonstrated reduced sympathetic outflow in patients with melanocortin-signaling deficiency. To confirm the role of the SNS in the regulation of renal glucose reabsorption, we treated both WT and ArcPOMC-deficient mice with epinephrine (0.3 mg/kg lean mass, i.p.) or saline and assessed rGLUT2 expression 60 min after the treatment. We found that epinephrine treatment increased rGLUT2 expression in the mutant as well as the WT mice (Figure 8B,C). Importantly, epinephrine treatment also abolished the differences in glucose tolerance (Figure 8D) between ArcPOMC-deficient and WT mice. Moreover, after epinephrine treatment, none of the mutant mice showed glycosuria at 60 and 120 min during OGTT (Supplementary Table 1B), supporting our hypothesis that the mutant mice show elevated glycosuria because of low sympathetic tone. These data corroborate a previous report (39) suggesting a role of the SNS in regulating renal glucose reabsorption and rGLUT2 in rats.
Discussion

In this study, we examined the role of hypothalamic POMC in the regulation of glucose homeostasis. Given the influence of genetics on energy homeostasis in mice (40), we assessed the phenotype of ArcPOMC-deficient mice on both C57BL/6 and 129S6 genetic backgrounds. Surprisingly, we found an improvement in glucose tolerance in ArcPOMC-deficient mice on both genetic backgrounds. Moreover, the mutant mice had normal fasting glycemia despite obesity and insulin resistance. These data are consistent with previous observations that MC4R deficient humans and mice exhibit normoglycemia despite obesity (12-14). Our data also suggest that hypothalamic POMC regulates plasma insulin levels and insulin sensitivity independently of changes in body weight, thereby supporting the observation by Fan et al. (13) that central melanocortins directly control serum insulin levels. Moreover, the findings that central melanocortin signaling regulates hepatic and muscle insulin sensitivity (41; 42) might explain the exacerbated insulin resistance in the ArcPOMC-deficient mice.

The FSIVGTT data showed that there was an increase in glucose effectiveness (insulin-independent glucose disposal) in ArcPOMC-deficient mice. This result supports a recent study by Morton et al. (30) that demonstrated a role of the brain in enhancing glucose tolerance independently of insulin action. We proposed that elevated glycosuria might explain the increased insulin-independent glucose disposal in ArcPOMC-deficient mice. Indeed, there was an increase in glycosuria in the mutant mice relative to their WT littermates. Elevated glycosuria in the absence of hyperglycemia in ArcPOMC-deficient mice suggests that hypothalamic POMC regulates the renal glucose threshold. The normal average renal glucose threshold for mice is 400
mg/dl (43), however ArcPOMC-deficient mice exhibited elevated glycosuria (Supplementary Table 1A) at average blood glucose levels of 230 and 180 mg/dl at 60 and 120 min, respectively, during OGTT (Figure 3E,F). MT II treatment decreased glycosuria in the mutant mice, indicating a direct role of the central melanocortin system in the regulation of glucose reabsorption. In contrast, Agrp increased glycosuria at 15 and 60 min, but not at 120 min, during OGTTs in WT mice. This discrepancy may be attributed to short-term Agrp treatment and/or a mechanism other than glycosuria, such as increased insulin sensitivity, that might be partly responsible for Agrp-mediated improvement in glucose tolerance.

We assessed rSGLT2 levels in ArcPOMC-deficient mice because glycosuria induced by pharmacological inhibition of rSGLT2 is a novel strategy to combat hyperglycemia in diabetes patients (31). Surprisingly, the mutant mice exhibited normal rSGLT2 levels. However, rGLUT2 was reduced in the mutant mice by 20%, indicating that GLUT2 deficiency mediates elevated glycosuria in ArcPOMC-deficient mice. Indeed, GLUT2 deficiency or gene mutations are linked to glycosuria in rodents (44) and humans even in the absence of hyperglycemia (45). We did not examine rSGLT2 or rGLUT2 intracellular trafficking in the mutant mice. It is possible that defective trafficking of the glucose transporters between cytoplasmic vesicles and the plasma membrane could be partly responsible for the elevated glycosuria in ArcPOMC-deficient mice. Future studies are needed to elucidate this possibility.

MT II and epinephrine abrogated the genotype differences in glucose tolerance and glycosuria, suggesting that suppressed sympathetic nervous system activity due to inadequate central melanocortin signaling is the underlying mechanism for elevated glycosuria in ArcPOMC-deficient mice. Our finding that epinephrine controls rGLUT2 levels and thus renal glucose reabsorption might explain, at least in part, reduced fasting glycemia and normal glucose
tolerance in the presence of insulin resistance in E/NE knockout mice (46). Moreover, the observation that MC4R-deficient mice (13) and humans (14) exhibit normal blood glucose levels despite obesity and insulin resistance might also be attributed to suppressed rGLUT2 expression since the subjects have decreased renal sympathetic tone (38; 47). Greenfield et al. have clearly demonstrated the presence of suppressed sympathetic activity and thus reduced blood pressure in obese MC4R-deficient subjects (38), thereby confirming the preclinical data reported in MC4R-knockout mice (47). Hence, it is possible that E/NE- and MC4R- deficient mice, like ArcPOMC-deficient mice in this study, exhibit exaggerated glycosuria because of suppressed rGLUT2 expression mediated by low sympathetic tone. Elevated glycosuria attributed to low sympathetic tone could also be one of the reasons why renal denervation improves glycemia in patients suffering from hypertension (48).

In summary, we have identified a previously unrecognized hypothalamic-SNS-renal axis (Figure 8E) that controls glucose homeostasis by regulating proximal tubular glucose reabsorption, and which complements other recently reported brain mediated insulin-independent mechanisms of glycemia regulation (30; 49). The SNS-mediated increased glucose reabsorption may be of physiological relevance during fight or flight responses to prevent glucose (energy) loss in urine and would complement other sympathetic actions on glucose homeostasis including inhibition of insulin secretion from beta cells and increased glucose production by the liver to meet increased metabolic demand under stress. This pathway (Figure 8E) also predicts that renal-specific antagonism of GLUT2 is an alternative, or possibly synergistic therapeutic approach to SGLT2 antagonism for diabetes control even in the presence of insulin resistance and obesity.
**Author Contributions.** K.H.C and M.J.L. conceived the study, designed experiments, analyzed results, and wrote the manuscript. M.R. discussed results and edited the manuscript. K.H.C, J.M.A., B.F., D.D.L., and N.Q performed experiments and edited the manuscript. M.J.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors have no conflict of interest relevant to this study.

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Figure Legends

Figure 1. Improved glucose tolerance despite insulin resistance in C57BL/6J background 24-week old obese ArcPOMC-deficient mice. (A) Body weight; (B) Fasting plasma insulin levels; (C, D) Insulin tolerance test (ITT); (E, F) Oral glucose tolerance test (OGTT). Bar graphs in C, D, E and F represent the corresponding area under the curves (AUC). Two-tailed Student’s t-test was used for comparisons. *P < 0.05, **P < 0.01, ***P < 0.001; n = 6. Error bars reflect mean ± s.e.m.

Figure 2. Evaluation of glucose homeostasis in 129S6/SvEvTac background 24-week old obese ArcPOMC-deficient mice. (A) Body weight; (B) Fasting plasma insulin levels; (C,D) Insulin tolerance test (ITT); (E, F) Oral glucose tolerance test (OGTT). Bar graphs in C, D, E and F represent the corresponding area under the curves (AUC). Two-tailed Student’s t-test was used for comparisons. *P < 0.05, **P < 0.01, ***P < 0.001; n = 6. Error bars reflect mean ± s.e.m.

Figure 3. Improved glucose tolerance in the presence of insulin resistance in 12-week old weight-matched ArcPOMC-deficient mice. (A) Body weight; (B) Fasting plasma insulin levels; (C, D) Insulin tolerance test (ITT) in weight-matched ArcPOMC-deficient mice; (E, F) Oral glucose tolerance test (OGTT) in weight-matched ArcPOMC-deficient mice. Bar graphs in C, D, E and F represent the corresponding area under the curves (AUC). Two-tailed Student’s t-test was used for comparisons. *P < 0.05; n = 6. Error bars reflect mean ± s.e.m.

Figure 4. Improved glucose tolerance assessed by frequently sampled intravenous glucose tolerance test (FSIVGTT) in ArcPOMC-deficient mice. (A, B) Blood glucose and corresponding area under the curves of 0-10 min data points in 8-week old ArcPOMC-deficient mice during FSIVGTT; (C) Plasma insulin levels and (D) Glucose effectiveness in 8-week old mice during
FSIVGTT. Two-tailed Student’s t-test was used for comparisons. \( *P < 0.05; \ n = 6 \) or 7. Error bars reflect mean \( \pm \) s.e.m.

**Figure 5.** Elevated glycosuria, normal liver glycogen levels, and normal gluconeogenesis in weight-matched or obese ArcPOMC-deficient mice. (A) 24 hr urine glucose concentration in weight-matched 12-week old mice before and after OGTT; (B) 24 hr urine glucose concentration in weight-matched 12-week old mice that were challenged with 10% glucose provided in their drinking water; (C) Fasting hepatic glycogen levels at baseline and post glucose challenge in weight-matched 8-week old mice, \( n=6 \); (D) Pyruvate tolerance test (PTT) in 8-week old weight-matched mice, \( n=6 \); (E) Pyruvate tolerance test in 16-week old obese mutant mice, \( n=6 \). Two-tailed Student’s t-test was used for comparisons. \( *P < 0.05, **P < 0.01; \ NS = not \ significant, \ P > 0.05; \ n = 6 \) or 7. Error bars reflect mean \( \pm \) s.e.m.

**Figure 6.** MTII and Agrp treatment in female ArcPOMC-deficient and WT mice, respectively. (A) OGTT in ArcPOMC-deficient and WT mice, bar graphs on the right represent AUC; (B) 24h body weight change after MTII or Agrp treatment; (C) 24h food intake. Two-tailed Student’s paired t-test was used for comparisons. \( **P < 0.01, ***P < 0.001; \ n = 6 \). Error bars reflect mean \( \pm \) s.e.m.

**Figure 7.** Reduced renal GLUT2 but not SGLT2 levels in ArcPOMC-deficient mice. (A,B) Representative Western blot images of renal cortical GLUT2 and SGLT2 respectively, \( n=8 \) per group for bar graphs that represent relative expression. Two-tailed Student’s t-test was used for comparisons. \( **P < 0.01 \). Error bars reflect mean \( \pm \) s.e.m.

**Figure 8.** ArcPOMC regulates renal GLUT2 via the sympathetic nervous system. (A) Whole kidney epinephrine (Epi) and norepinephrine (Nor-epi) levels, \( n=6 \); (B) Renal cortical GLUT2
expression in the presence of epinephrine treatment in ArcPOMC-deficient mice; (C) Renal cortical GLUT2 expression in the presence of epinephrine treatment in WT mice, n=8 per group for bar graphs that represent relative expression; (D) Oral glucose tolerance test (OGTT) in epinephrine (Epi) or saline treated mice, n=6 per group, genotype effect: $F[1,23] = 20.2$, $P = 0.0002$, and treatment effect: $F[1,23] = 84.9$, $P < 0.0001$; (E) Proposed insulin-independent mechanism by which hypothalamic POMC-deficiency improves glucose tolerance. Two-tailed Student’s t-test or two-way ANOVA, followed by Bonferroni’s multiple comparisons test, were used for comparisons. **$P < 0.01$. Error bars reflect mean ± s.e.m.
Body Weight (g)

Plasma Insulin (ng/ml)

Blood glucose (mg/dl)

AUC (mg/dl × min)

Obese Male

Obese Female

ITT, Obese Male

ITT, Obese Female

OGTT, Obese Male

OGTT, Obese Female

AUC (mg/dl × min)
A. Urine glucose (mg/dl)

10% glucose challenge

WT ArcPOMC KO

Log Urine glucose (mg/dl)

WT ArcPOMC KO

Liver Glycogen (mg/mg tissue)

Baseline

Post glucose challenge

NS

B. Weight-matched Male

10% glucose challenge

Weight-matched Female

Log Urine glucose (mg/dl)

WT ArcPOMC KO

Liver Glycogen (mg/mg tissue)

Baseline

Post glucose challenge

NS

C. Weight-matched Male

10% glucose challenge

Weight-matched Female

Log Urine glucose (mg/dl)

WT ArcPOMC KO

Liver Glycogen (mg/mg tissue)

Baseline

Post glucose challenge

NS

D. PTT, Weight-matched Male

Blood glucose (mg/dl)

Time (min)

E. PTT, Male

Blood glucose (mg/dl)

Time (min)

D. PTT, Weight-matched Female

Blood glucose (mg/dl)

Time (min)

E. PTT, Female

Blood glucose (mg/dl)

Time (min)
A

Blood glucose (mg/dl) vs. Time (min)

- ArcPOMC KO, Saline
- ArcPOMC KO, MTII
- WT, Saline
- WT, Agrp

AUC (mg/dl.min)

WT

ArcPOMC KO

Saline MTII Saline Agrp

24 h Food Intake (g)

- ArcPOMC KO
- WT

24 h Δ Body Weight (g)

- ArcPOMC KO
- WT

B

C

Diabetes
A. Relative GLUT2 expression

B. Relative SGLT2 expression

Diabetes
**A** Relative GLUT2 expression (arbitrary unit) across different conditions.

**B** Western blots showing GLUT2 expression levels in ArcPOMC KO and WT mice after saline or epinephrine treatment.

**C** Graphs depicting relative GLUT2 expression levels in WT and ArcPOMC KO mice under saline and epinephrine treatment.

**D** OGTT (oral glucose tolerance test) showing blood glucose levels over time for WT, ArcPOMC KO, and their respective saline and epinephrine treatments.

**E** Diagram illustrating the relationship between POMC, SNS activity, renal epinephrine, GLUT2 expression, and improved glucose tolerance in ArcPOMC KO mice.
Supplementary Figure 1. Elevated glycosuria, which is reversed after epinephrine treatment, in weight-matched ArcPOMC-deficient mice during oral glucose tolerance (OGTT). (A) Semi-quantitative urine glucose levels during OGTT, n=12; (B) Semi-quantitative urine glucose levels during OGTT in epinephrine treated mice, n=14.

Supplementary Figure 2. MT II reverses glycosuria in ArcPOMC-deficient mice and Agrp induces glycosuria in WT mice. (A) Semi-quantitative urine glucose levels during OGTT after MT II or saline treatment in ArcPOMC-deficient mice, n=12; (B) Semi-quantitative urine glucose levels during OGTT after Agrp or saline treatment in WT mice, n=12.
### A

| Urine Glucose (mg/dl) | 15 min | 30 min | 60 min | 120 min |
|-----------------------|--------|--------|--------|---------|
|                       | WT     | Mutant | WT     | Mutant  | WT     | Mutant  | WT     | Mutant  |
| 100                   | 0      | 0      | 0      | 0       | 0      | 0       | 0      | 0       |
| 250                   | 7      | 0      | 8      | 0       | 0      | 0       | 0      | 5       |
| 500                   | 5      | 0      | 0      | 8       | 0      | 12      | 0      | 7       |
| 1000                  | 0      | 5      | 0      | 4       | 0      | 0       | 0      | 0       |
| >2000                 | 0      | 7      | 0      | 0       | 0      | 0       | 0      | 0       |

A number of weight-matched mice that manifested glycosuria during OGTT.

### B

| Urine Glucose (mg/dl) | 15 min | 30 min | 60 min | 120 min |
|-----------------------|--------|--------|--------|---------|
|                       | WT     | Mutant | WT     | Mutant  | WT     | Mutant  | WT     | Mutant  |
| 100                   | 8      | 12     | 11     | 9       | 0      | 0       | 0      | 0       |
| 250                   | 6      | 2      | 3      | 5       | 0      | 0       | 0      | 0       |
| 500                   | 0      | 0      | 0      | 0       | 0      | 0       | 0      | 0       |
| 1000                  | 0      | 0      | 0      | 0       | 0      | 0       | 0      | 0       |
| >2000                 | 0      | 0      | 0      | 0       | 0      | 0       | 0      | 0       |

B number of mice that manifested glycosuria during OGTT in the presence of epinephrine.
| Urine Glucose (mg/dl) | 15 min. | 60 min. | 120 min. |
|----------------------|---------|---------|----------|
|                      | Saline  | MT II   | Saline   | MT II   | Saline  | MT II   |
| 100                  | 0       | 0       | 0        | 6       | 0       | 0       |
| 250                  | 0       | 6       | 0        | 0       | 2       | 0       |
| 500                  | 4       | 0       | 1        | 0       | 3       | 0       |
| 1000                 | 2       | 0       | 5        | 0       | 0       | 0       |
| >2000                 | 0       | 0       | 0        | 0       | 0       | 0       |

| Urine Glucose (mg/dl) | 15 min. | 60 min. | 120 min. |
|----------------------|---------|---------|----------|
|                      | Saline  | Agrp    | Saline   | Agrp    | Saline  | Agrp    |
| 100                  | 3       | 0       | 4        | 0       | 0       | 0       |
| 250                  | 3       | 6       | 2        | 5       | 0       | 0       |
| 500                  | 0       | 0       | 0        | 1       | 0       | 0       |
| 1000                 | 0       | 0       | 0        | 0       | 0       | 0       |
| >2000                 | 0       | 0       | 0        | 0       | 0       | 0       |