Targeted disruption of Slc19a2, the gene encoding the high-affinity thiamin transporter Thtr-1, causes diabetes mellitus, sensorineural deafness and megaloblastosis in mice

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Thiamin-responsive megaloblastic anemia syndrome (TRMA) is characterized by diabetes mellitus, megaloblastic anemia and sensorineural deafness. Mutations in the thiamin transporter gene SLC19A2 cause TRMA. To generate a mouse model of TRMA, we developed an Slc19a2 targeting construct using transposon-mediated mutagenesis and disrupted the gene through homologous recombination in embryonic stem cells. Erythrocytes from Slc19a2/C0/C0 mice lacked the high-affinity component of thiamin transport. On a thiamin-free diet, Slc19a2/C0/C0 mice developed diabetes mellitus with reduced insulin secretion and an enhanced response to insulin. The diabetes mellitus resolved after 6 weeks of thiamin repletion. Auditory-evoked brainstem response thresholds were markedly elevated in Slc19a2/C0/C0 mice on a thiamin-free diet, but were normal in wild-type mice treated on that diet as well as thiamin-fed Slc19a2/C0/C0 mice. Bone marrows from thiamin-deficient Slc19a2/C0/C0 mice were abnormal, with a megaloblastosis affecting the erythroid, myeloid and megakaryocyte lines. Thus, Slc19a2/C0/C0 mice have provided new insights into the TRMA disease pathogenesis and will provide a tool for studying the role of thiamin homeostasis in diabetes mellitus more broadly.

INTRODUCTION

Thiamin-responsive megaloblastic anemia syndrome (TRMA; OMIM 249270) is an autosomal recessive disorder, characterized by diabetes mellitus, megaloblastic anemia and sensorineural deafness (1–4). Pharmacological oral thiamin ameliorates the anemia, may decrease insulin requirements, and appears to arrest hearing loss (5–8). We and others previously identified SLC19A2, which encodes the high-affinity thiamin transporter THTR-1, as the TRMA disease gene (9–12).

While the molecular basis of TRMA has been established, its pathogenesis is only partially understood. Biochemical studies of TRMA erythrocytes and skin fibroblasts showed that the high-affinity, saturable component of thiamin transport was lacking (8,13). Consistent with this transport defect, intracellular thiamin concentrations and the activities of thiamin-dependent enzymes are low in TRMA erythrocytes (8,14). Thus, a deficit of intracellular thiamin appears to be critical for disease pathogenesis.

The specificity of the phenotype associated with TRMA is striking. Thiamin is an important cofactor for enzymes such as the pyruvate dehydrogenase complex and 2-ketoglutarate dehydratase. Nearly all cells require these enzymes for the Krebs cycle, but the loss of THTR-1 only affects a restricted set of tissues. Also, there are marked disparities between the expression pattern of SLC19A2 and the tissues affected in TRMA (9–11). Even more perplexing, there is limited clinical overlap between TRMA and diseases resulting from dietary thiamin deficiency: beriberi and Wernicke–Korsakoff syndrome. Thus, a fuller understanding of TRMA disease pathogenesis could provide novel insights into the role of thiamin and thiamin transport in diseases affecting glucose homeostasis, hearing and hematopoiesis.

To facilitate the study of TRMA, we generated a mouse model of SLC19A2 deficiency. Recently, our group cloned and characterized the mouse orthologue, Slc19a2 (15), which encodes a bona fide high-affinity thiamin transporter (16,17). Here, we report that targeted disruption of Slc19a2 in mice...
results in defect in a saturable component of thiamin transport in erythrocytes. Both heterozygous (Slc19a2+/−) and homozygous (Slc19a2−/−) mice were normal when fed standard chow. When challenged with a thiamin-deficient diet, the Slc19a2−/− mice developed diabetes mellitus, sensorineural deafness and megaloblastosis. All aspects of the TRMA phenotype were reversible when thiamin was repleted. Characterization of the diabetes mellitus revealed a blunted secretion of insulin but preserved end-organ sensitivity to insulin. The Slc19a2-deficient mice are an excellent model of TRMA, and provide a novel reagent for studying the role of thiamin in diabetes mellitus.

RESULTS

Generation of Slc19a2−/− mice

Using an Slc19a2 targeting vector generated through transposon-mediated mutagenesis (Fig. 1A), we generated seven targeted embryonic stem (ES) cell lines (Fig. 1B). Highly chimeric founders that transmitted the knockout allele were obtained after microinjection of two lines, and the allele was bred to homozygosity (Fig. 1C). Slc19a2−/− mice had no normal Slc19a2 transcript but an aberrantly spliced out-of-frame transcript that lacked a 3’ portion of exon 2 (Fig. 1D).

Thiamin uptake in erythrocytes

To document that the Slc19a2− allele had obliterated the high-affinity transport of thiamin, we measured thiamin uptake in erythrocytes from Slc19a2+/+ , Slc19a2+/− and Slc19a2−/− mice (n=4 for each genotype). The profile for thiamin transport in the Slc19a2+/− and Slc19a2−/− erythrocytes was biphasic, with a nonlinear component at lower thiamin concentrations (0.1–0.5 μM) and a linear one at higher concentrations (Fig. 2). This profile replicated the pattern observed with human erythrocytes, for which there are high-affinity saturable and low-affinity non-saturable components (8,18). Slc19a2+/− erythrocytes lacked the high-affinity component but retained the non-saturable one (Fig. 2). At thiamin concentrations of 0.1 and 0.5 μM, uptake was significantly decreased in Slc19a2−/− erythrocytes compared with Slc19a2+/+ cells (0.1 μM: P < 0.0001; 0.5 μM: P < 0.0001). Thiamin uptake for the Slc19a2+/- erythrocytes was intermediate between those for Slc19a2−/− and Slc19a2+/. This pattern of thiamin uptake faithfully reproduced the findings observed in TRMA patients and carriers (8,18).

Initial phenotype assessment

The Slc19a2−/− and Slc19a2+/− mice appeared to be normal when maintained on standard mouse chow, and had normal spot blood sugar levels and complete blood counts (CBCs). Plasma thiamin levels were not significantly different between the Slc19a2−/− and Slc19a2+/+ mice (646 ± 51 nM versus 831 ± 82 nM) (Fig. 3).

Since standard mouse chow contains a substantial amount of thiamin, we considered the possibility that this diet was obfuscating adverse effects of the Slc19a2− allele. To test this, mice were challenged with a thiamin-free diet. For Slc19a2+/- mice, ataxia developed after 4–5 weeks on this diet and death occurred shortly thereafter. In contrast, the Slc19a2−/− mice died at 3–4 weeks into the challenge without ataxia. Plasma thiamin levels were not significantly different between Slc19a2−/− and Slc19a2+/+ mice (24 ± 6 nM versus 27 ± 3 nM), although they were significantly lower than when mice were fed standard chow (Fig. 3).

Diabetes mellitus

Since hyperglycemia was noted in challenged Slc19a2−/− mice prior to death, glucose tolerance testing (GTT) was performed after 17 days of thiamin withdrawal. The fasting blood glucose level was significantly elevated in Slc19a2−/− compared with wild-type mice (211 ± 36 mg/dl versus 88 ± 4 mg/dl; P < 0.005). The peak level was observed at 60 minutes (Fig. 4A), and the blood glucose was still significantly elevated in Slc19a2−/− compared with Slc19a2+/+ mice (394 ± 71 mg/dl versus 99 ± 8 mg/dl; P < 0.001) at 120 minutes, which is the usual time point for terminating GTT. An additional time point at 180 minutes revealed persistent hyperglycemia in the Slc19a2−/− mice that was above their fasting baseline. The glucose intolerance resolved when the Slc19a2−/− mice were refed a thiamin-replete diet for 6 weeks (data not shown).

To characterize the type of diabetes mellitus in the Slc19a2−/− mice, we performed an insulin secretion test (IST) using an intravenous injection of glucose and an insulin tolerance test (ITT) using an intraperitoneal injection of insulin. On a normal diet, insulin secretion in Slc19a2−/− mice was not significantly different from that of the wild-type animals (data not shown). In the thiamin-deficient state, insulin secretion was significantly impaired in Slc19a2−/− mice compared with Slc19a2+/+ mice (0.08 ± 0.05 ng/ml versus 0.41 ± 0.14 ng/ml at 15 minutes; P < 0.05) (Fig. 4B). ITTs, performed in the thiamin-deficient state, revealed a greater and prolonged hypoglycemic response in Slc19a2−/− mice than in Slc19a2+/+ mice (77% versus 36% at 60 minutes) (Fig. 4C).

We performed immunohistological analysis of pancreata from Slc19a2−/− and Slc19a2+/+ mice in order to determine whether the loss of insulin secretion was associated with a gross anatomical disruption, but observed no discernible differences in islet structure, total pancreatic islet area or the α to β-cell ratios between Slc19a2−/− and Slc19a2+/+ mice (Fig. 4D). Therefore, the diabetic phenotype with reduced insulin secretion observed in the Slc19a2−/− mice cannot be attributed to a reduction in β-cell mass or disorganization of the islet structure.

Auditory function

When maintained on standard chow, Slc19a2−/− mice showed a normal auditory-evoked brainstem response (ABR) to different acoustic stimuli (click, 8, 16 and 32 kHz) that span the sensitive hearing range of mice (Fig. 5A). Waveforms with a characteristic series of four or five peaks were detected in response to normal stimulus intensities (Fig. 5B). On a thiamin-free diet, however, Slc19a2−/− mice showed a striking loss of hearing, while the wild-type controls continued to have normal ABR thresholds (Fig. 5A). For example, the wild-type mice
Figure 1. Targeted disruption of Slc19a2. (A) Maps of the Slc19a2 allele, targeting vector and the resulting targeted allele. The open and filled boxes indicate the neomycin-resistance gene and Slc19a2 exons, respectively. X indicates an XbaI site. (B) Southern analysis of genomic DNA digested with XbaI from wild-type (+/+) knockout (-/-) and heterozygous (+/-) mice. (C) Genotyping by PCR. (D) Northern analysis with total liver RNA from wild-type and knockout mice. Reprobing with β-actin revealed that the knockout lane was substantially overloaded related to the wild type. In the knockout, the 3.0 kb band corresponds to an aberrantly spliced transcript lacking exon 2 with the neoR. It was confirmed by cloning and sequencing.
activity in the cell (Ci) and that in the medium (Cm). (B) Click stimulus, while no significant waveform could be detected in a knockout mouse, even using the maximum stimulus intensity of 100 dB SPL possible with the apparatus (Fig. 5B). Some Slc19a2−/− mice had weak ABR waveforms, but with very elevated thresholds. When detected, these waveforms were atypical, with irregular peaks and prolonged peak latencies.

To investigate whether the auditory phenotype was reversible, we re-fed three mice a thiamin-replete diet. This led to a recovery of auditory function in two mice, which showed thresholds of 40–48 dB SPL for the click stimulus. The third mouse showed no recovery of hearing, with undetectable responses even at 100 dB SPL.

Histological analysis of cochleae from Slc19a2−/− and Slc19a2+/+ mice revealed no overt differences in the sensorineural epithelium, tectorial membrane, stria vascularis or spiral ganglion (Fig. 5C).

Bone marrow

CBCs and peripheral blood smears were not different between the Slc19a2−/− and Slc19a2+/+ mice maintained on a thiamin-free diet. When we examined their bone marrows, we observed dysplastic hematopoiesis with a reversed myeloid-to-erythroid (M:E) ratio due to expansion of the erythroid component in the knockout but not the wild-type mice (Fig. 6A and B). In the erythroid lineage, Slc19a2−/− marrows had a dysynchrony of nuclear and cytoplasmic maturation, with a lag in nuclear maturation (Fig. 6F and G). There was an increase of early erythroid precursors, resulting in relative erythroid hyperplasia. In all stages, the size of the cells was greater than in the wild-type cells. Nuclear chromatin was uneven (sliced salami appearance), with an immature appearance (Fig. 6B). Some of the polychromatophilic erythroblasts were twice as large as normal ones (Fig. 6E–G). Evidence of ineffective erythropoiesis was apparent from the fragmentation and condensation of the erythroid nuclei. In the myeloid lineage, Slc19a2−/− metamyelocytes and bands were much larger than their wild-type counterparts (Fig. 6C and D), reminiscent of the giant white blood cell precursors reported in other megaloblastic processes (19). Increased lobulation in megakaryocyte nuclei was also present. As expected, lymphocytes and plasma cells were spared the cellular gigantism and nuclear cytoplasm asynchrony. These bone marrow changes in the Slc19a2−/− mice were consistent with a megaloblastic process.

DISCUSSION

In this report, we describe the generation of a mouse lacking the high-affinity thiamin transporter, Thtr-1, encoded by Slc19a2. While erythrocytes and, presumably, other cells in these mice lack this high-affinity transporter, the Slc19a2−/− mice had no overt phenotype until challenged with a thiamin-deficient diet. On that diet, they developed diabetes mellitus, profound hearing loss and megaloblastosis in the bone marrow. Thus, the Slc19a2−/− mouse is a bona fide model of TRMA.

Patients with TRMA present in infancy or early childhood despite diets that contain thiamin, while the knockout mice were asymptomatic on their normal diet. There are several factors contributing to this discrepancy. First, a comparison of the thiamin-uptake curves for TRMA and Slc19a2−/− erythrocytes reveals that the former have much lower uptake at a given thiamin concentration (e.g. Ci/Cm at 0.1 μM of thiamin: human 0.08–0.1 versus mouse 0.71) (8,18). Second, the normal plasma concentration of thiamin is far lower in humans than in mice (0.03 μM versus 0.83 μM), making low-affinity transport much less effective. Finally, the ratio of average thiamin intake to minimal requirement is lower in humans than mice (20,21). The typical diet for persons in the USA contains only slightly more than the 1.5 mg RDA. In contrast, we found that wild-type mice could tolerate reductions in the thiamin concentration of their chow from 16 mg/kg to 1.375 mg/kg without developing ataxia or other symptoms. While mice have a higher basal metabolic rate than humans, implying a greater

**Figure 2.** Thiamin uptake by Slc19a2−/− (diamonds), Slc19a2+/− (triangles) and Slc19a2+/+ (squares) erythrocytes. (A) Ratio between the [3H]thiamin radioactivity in the cell (Ci) and that in the medium (Cm). (B) Thiamin uptake of 25 μl of erythrocytes at different concentrations of thiamin in the medium.
need for thiamin, mice lacking Thtr-1 should have higher thiamin concentrations intracellularly when maintained on their usual diet than TRMA patients.

Thiamin pyrophosphate, the active form of the vitamin, is a coenzyme for several enzymes that play pivotal roles in energy metabolism. These include pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and branched-chain ketoacid dehydrogenase complexes. It has been shown that a deficiency of intracellular thiamin results in an impairment in the aerobic oxidative synthesis of ATP (22,23). Experimental thiamin deficiency is also a classic model of oxidative stress that results in functional and morphological cell damage. Thus, it is striking that the TRMA phenotype overlaps with several mitochondrial diseases (24). This suggests that loss of oxidative metabolism is a fundamental part of the TRMA phenotype. Future studies with the Slc19a2−/− mice will be directed towards pinpointing the metabolic abnormalities in vivo in tissues lacking THTR-1.

Slc19a2−/− mice have abnormal hematopoiesis with megaloblastic anemia when maintained on a thiamin-free diet. Megaloblastic anemias are associated with mitochondrial disorders (e.g. Pearson syndrome) but also diseases with deranged DNA/RNA synthesis (e.g. vitamin B12 and folate deficiencies). This latter association may be relevant for TRMA, since thiamin deficiency may also reduce DNA and RNA synthesis through the inhibition of the production of the nucleic acid precursor ribose. Ribose is synthesized de novo predominantly through the non-oxidative pentose phosphate pathway, utilizing the thiamin-dependent enzyme transketolase (25). Recently, Neufeld and colleagues (26) documented that TRMA fibroblasts have reduced non-oxidative ribose synthesis, leading them to propose that inadequate ribose 5-phosphate synthesis is a plausible explanation for the abnormal erythropoiesis. Previous work by Haworth and colleagues (4) with marrow aspirates from TRMA patients had suggested that DNA/RNA synthesis generally, and pyrimidine synthesis specifically, was normal (4). Of note, they used thiamin-containing medium for culturing the TRMA marrow cells, so may have unwittingly obscured relevant metabolic derangements. This would correlate well with the fact that the hematopoietic abnormalities constitute the aspect of TRMA that is consistently improved with thiamin therapy. In sum, the megaloblastic anemia associated with TRMA (and its correlate in Slc19a2−/− mice) may arise from abnormal oxidative metabolism and/or reduced ribose synthesis.

Slc19a2−/− mice developed profound sensorineural hearing loss when maintained on the thiamin-deficient diet. The precise anatomical site perturbed in TRMA (e.g. cochlea, VIIIth nerve) has not been identified, although we excluded a gross structural defect in the cochlea. The reversibility of the hearing loss, however, suggested a metabolic dysfunction. The hearing loss was so profound that no localization could be inferred from the wave pattern on ABR. Previous studies with rats subjected to experimental thiamin deficiency revealed modest ABR abnormalities at day 24 or later (27). In those studies, the interpeak latencies between waves I and III as well as between I and IV increased, while the I–II interpeak latency was normal. Based on the fact that wave II is most strongly related to the brainstem cochlear nuclei, those authors concluded that the adverse effects of thiamin deficiency were in the VIIIth cranial nerve or brainstem. Similar results were obtained when ABRs were administered to alcoholic patients with and without Wernicke–Korsakoff syndrome (28). That said, the data about hearing loss from thiamin-deficiency states may not apply to TRMA and Slc19a2−/− mice. First, Thtr-1 has been immuno-localized to hair cells in the cochlea (17 and our unpublished results), a cell type that is sensitive to metabolic disturbances. Second, dietary thiamin deficiency is associated with a host of central and peripheral nervous systems deficits, while similar neurological problems have not been highly prevalent in TRMA. Further studies, perhaps with more modest thiamin deprivation, might elucidate the source of the sensorineural hearing loss in TRMA.

Perhaps the most intriguing aspect of TRMA, and the one with the greatest general importance, is the development of diabetes mellitus. Diabetes mellitus has been present at diagnosis in nearly all patients with TRMA. In some affected individuals, particularly those diagnosed at young age, thiamin therapy was a successful palliation. A study of two such patients revealed that the diabetes mellitus returned 7–10 years later (7). GTTs performed when they became insulin-dependent revealed markedly inadequate insulin secretion. Hyposecretion of insulin was also noted in Slc19a2−/− mice when they became diabetic.

Sundaresen and co-workers (29–32) explored the role of thiamin in pancreatic function using thiamin-deficient rats. They documented that these rats developed hyperglycemia and had decreased insulin secretion basally and in response to several provocative agents. Proinsulin production in thiamin-deficient rats was normal basally but decreased in response to a sulfonylurea, a known insulin secretagogue. Oxidative metabolism of glucose and pyruvate were decreased significantly in isolated pancreatic islet cells compared with controls. Taken together, their work showed the production and secretion of insulin is abnormal when intracellular thiamin is inadequate in β-islet cells.

The response of peripheral tissues does not appear to contribute to the etiology of diabetes mellitus in thiamin deficiency. First, the utilization of glucose by peripheral tissues does not decrease. In fact, studies of brain metabolism have
revealed increased flux through the glycolytic pathway in thiamin deficiency (33,34). In thiamin-deficient rats that were hyperglycemic, the hypoglycemic response to insulin administration was normal, as was the response to an insulin secretagogue (30). Similarly, blood glucose levels were reactive to insulin in thiamin-deficient Slc19a2/C0/C0 mice. We conclude, therefore, that insulin production and/or secretion are the principal problem leading to diabetes mellitus in TRMA.

There appears to be a connection between thiamin and the risk of complications from diabetes mellitus. In particular, cells cultured in high glucose concentrations replicate more slowly and have increased quantities of advanced glycation end-products (35–38). Both abnormalities were reversed when thiamin concentrations were increased in the media. While there is no compelling reason to believe that decreased intracellular thiamin is the principal cause of diabetes mellitus, there are data suggesting that the levels of this vitamin impact on disease severity and complications. Future studies with the Slc19a2 allele, such as the use of heterozygous mice induced genetically or pharmacologically to become diabetic, may provide novel insights into the role of intracellular thiamin deficiency in the course of diabetes mellitus.

MATERIALS AND METHODS

Generation of Slc19a2 knockout mice

A bacterial artificial chromosome clone that contained the mouse gene Slc19a2 was identified (15). A 9.6 kb genomic DNA fragment, that contained exon 2 and its flanking introns was cloned into the pKO Scrambler NTKV vector (Stratagene, La Jolla, CA). A neoR cassette was inserted into exon 2 using transposon-mediated insertional mutagenesis. EZ::TN pMOD Transposon (Epiconcert Technologies, Madison, WI, USA) was employed for construction of the transposon. 129/SvJ ES cells

Figure 4. Glucose tolerance, insulin secretion, and insulin tolerance testing. (A) GTT in Slc19a2−/− (diamonds, n = 9) and Slc19a2+/-+ (squares, n = 9) mice on thiamin-free diet at day 17. (B) IST in Slc19a2−/− and Slc19a2+/-+ mice on thiamin-free diet at day 14. Black and white bars indicate knockout (n = 3) and wild-type (n = 3) mice, respectively. (C) ITT in Slc19a2−/− (diamonds, n = 2) and Slc19a2+/-+ (squares, n = 4) mice on thiamin-free diet at day 16. Fasting glucose levels are 100% at 0 min. (D) Immunostaining of pancreatic islet cells with anti-insulin and anti-glucagon antibodies.
Figure 5. ABR thresholds in Slc19a2<sup>−/−</sup> and Slc19a2<sup>+/+</sup> mice. (A) Responses to click, 8, 16 and 32 kHz stimuli are shown. The white and black bars indicate regular and thiamin-free diets, respectively. (B) Waveforms for click stimulus on a 4 μV fixed scale for comparison are shown. (C) Hematoxylin–eosin staining of cochleae from Slc19a2<sup>−/−</sup> and Slc19a2<sup>+/+</sup> mice on a thiamin-free diet.
Figure 6. Bone marrow findings of Slc19a2<sup>−/−</sup> and Slc19a2<sup>+/+</sup> mice on a thiamin-free diet. (A) Normal marrow from a wild-type mouse. (B) A reversed M:E ratio was found in the knockout mice. A fragmented erythroblast nucleus is indicated (arrow). (C) Normal band form a wild-type marrow. (D) Giant metamyelocytes and bands in knockout marrow. (E) Normal polychromatophilic erythroblast (arrow) from a wild-type marrow. (F and G) Dyssynchrony of the nucleus and cytoplasm is observed in the knockout marrows. The cytoplasms of polychromatophilic erythroblasts are larger than wild-type counterparts but have immature nuclei. Evidence of ineffective erythropoiesis is apparent.
were electroporated with the linearized targeting vector. Genomic DNAs from G418-selected ES cell clones were screened using long-range PCR (Roche Molecular Biochemicals, Indianapolis, IN, USA) with a forward primer matching the neo\(^\text{R}\) gene (F: 5'-AACAGATGGCTGGCAACTCAGAAGG-3') and a reverse primer matching exon 3 (R: 5'-CGGGACGAATAACACAT-CAGGAAG-3') (Fig. 1A). Southern analysis was used to confirm the homologous recombinant events. Two independently targeted cell lines were microinjected into C57BL/6 blastocyst. Highly chimeric founders that transmitted the targeted allele were obtained, and the allele was bred to homozygosity. For PCR-based genotyping, we used a primer pair (F1: 5'-CTCGTCTGCAAGTTCATCTA-3' and R1: 5'-AGACAATCGGCTCTGCTGAT-3') to detect the targeted allele and another set (F2: 5'-TTACCT-GTGCTGCTGTGTTTC-3' and R2: 5'-GATGGTTAGCTGCT-GGGTGTA-3') for the wild-type allele (Fig. 1A and C). Total liver RNAs were extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) and northern analysis was performed with a partial Slc19a2 cDNA probe (15).

### Assessment of thiamin uptake

Blood was obtained from the inferior vena cavae of 16- to 18-week-old mice. Samples were washed with HBS buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\) and 5 mM glucose, pH 7.4) and spun at 3000 g three times, and then the erythrocytes were collected. Cells (25 \(\mu\)l) were incubated for 30 min at 37°C in 250 \(\mu\)l of HBS containing different concentrations of \[^3\text{H}\]thiamin hydrochloride as described previously (8). To terminate the reaction, ice-chilled HBS was added. Erythrocytes were centrifuged at 12 000 g for 30 s and washed three times with cold HBS. Proteins were precipitated with 10% (w/v) trichloroacetic acid, and the radioactivity in the supernatants was measured using a scintillation counter. Thiamin uptake was reported in pmol/\(\mu\)l of intracellular water, which was determined as described previously (39).

### Thiamin-free diet challenge

Mice were maintained routinely with Picolab Mouse Diet 20 (Lab Diet, Brentwood, MO), which has a thiamin concentration of 16 mg/kg. To challenge the mice, the Thiamin Deficient Diet (ICN Biomedicals, Inc., Aurora, OH) (0 mg/kg of thiamin) was used. For thiamin repletion, Thiamin Control Diet (22 mg/kg of thiamin) (ICN) was used. Mice were fed \textit{ad libitum}, except for mice undergoing IST and ITT, which were pair-fed. Plasma thiamin concentrations of mice were measured at LabCorp (Research Triangle Park, NC).

### GTT, IST and ITT

\(\text{Slc19a2}^{+/+}\) and \(\text{Slc19a2}^{−/−}\) mice, aged 14–16 weeks, were used. For GTT and IST, mice were fasted for at least 16 h prior to the study. For ITT, mice were fasted for at least 6 h prior to the studies. GTT was performed using an intraperitoneal injection of glucose (1 g/kg), and blood samples were collected from the tail vein immediately before and 15, 30, 60, 90, 120 and 180 min after injection. For the IST, glucose (1 g/kg) was injected intravenously at day 14 on a thiamin-free diet. In addition to the time points used for the GTT, an additional 5 min time point was assessed. ITT was performed with an intraperitoneal injection of porcine insulin (0.75 U/kg (Sigma-Aldrich, St Louis, MO) at day 16 on a thiamin-free diet and blood sampling was done as per the GTT protocol. Blood glucose levels were measured using an Elite XL glucometer (Bayer, Mishawaka, IN). Plasma insulin levels were measured with the Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem, Chicago, IL).

### ABR

Hearing testing was performed in males, aged 14–16 weeks, that had been anesthetized with Avertin (0.25 mg/g body weight), using the SmartEP ABR system, version 2.1 (Intelligent Hearing Systems, Miami, FL) essentially as described previously (40,41). Reference and ground electrode needles were placed subcutaneously at the vertex, ventrolateral to the left ear and ventrolateral to the right ear, respectively. Binaural stimulation (click, 8, 16 or 32 kHz) was presented at a rate of 25/s and averaged across 512 sweeps. Responses were bandpass-filtered below 100 and above 3000 Hz and amplified 100 000 times. The response threshold was defined as the minimal sound pressure level (SPL) that produced a characteristic waveform. Stimulation began at 90 dB SPL and decreased in 10 dB steps. When threshold was approached, step changes were reduced to 5 dB and then 3 dB SPL. ABR thresholds were detected as visually recognizable waveforms on a normalized scale.

### Histology

Pancreata were fixed in 10% formalin and embedded in paraffin. Sections (4 \(\mu\)m) were mounted on glass slides and stained with hematoxylin–eosin (H–E). For detection of insulin and glucagon, sections were stained with a guinea pig polyclonal anti-swine insulin antibody and a rabbit anti-glucagon antibody (Dako, Carpinteria, CA), respectively. The immunoreactivity was detected using the Multi-link-HRP Supersensitive System (BioGenex, San Ramon, CA).

Cochleae were fixed with 2% paraformaldehyde and 3% glutaraldehyde in PBS for 24 h and decalcified in 50 mM EDTA in PBS for 3 weeks at 4°C. Samples were embedded in paraffin blocks. Paraffin sections (8 \(\mu\)m) were deparaffinized, rehydrated and then stained with H–E.

 Femurs of mice were dissected away from the soft tissues. Both ends of the femurs were cut off and the bone marrow samples were harvested from the cavities using 0.4 ml of buffered PBS. These bone marrow samples were diluted further with PBS and then placed on slide glasses using Cytospin 3 (Shandon, Pittsburgh, PA). Giemsa staining was performed for the bone marrow cytospins and peripheral blood smears.

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