Metabolic, Endocrine and Genitourinary Pathobiology

Development of Diabetes in Mice with Neuronal Deletion of Shp2 Tyrosine Phosphatase

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Obesity and diabetes, termed “diabetesity,” are serious health problems that are increasing in frequency. However, the molecular mechanisms and neuronal regulation of these metabolic disorders are not fully understood. We show here that Shp2, a widely expressed Src homology 2-containing Tyr phosphatase, plays a critical role in the adult brain to control food intake, energy balance, and metabolism. Mice with a neuron-specific, conditional Shp2 deletion were generated by crossing a pan-neuronal Cre-line (CRE3) with Shp2^{flox/flox} mice. These congenic mice, CRE3/Shp2-KO, developed obesity and diabetes and the associated pathophysiological complications that resemble those encountered in humans, including hyperglycemia, hyperinsulinemia, hyperleptinemia, insulin and leptin resistance, vasculitis, diabetic nephropathy, urinary bladder infections, prostatitis, gastric paresis, and impaired spermatogenesis. This mouse model may help to elucidate the molecular mechanisms that lead to the development of diabetes in humans and provide a tool to study the *in vivo* complications of uncontrolled diabetes. (Am J Pathol 2008, 172:1312–1324; DOI: 10.2353/ajpath.2008.070594)

The recently adopted term “diabetesity”¹ reflects the escalating rates of both obesity and diabetes in developed and developing countries during the past 2 decades. According to World Health Organization, 8% (~300 million) of adults worldwide are extremely obese, as defined by a body mass index of >30 kg/m² (reviewed in Ref. 1). Obesity decreases life expectancy between 3 and 13 years,² and it constitutes one of the main causes of morbidity and mortality worldwide. In the United States,

approximately 300,000 deaths a year are currently attributed to weight gain and obesity (reviewed in Ref. 2). Obesity is the most common cause of insulin resistance in humans, leading to the failure of insulin to stimulate glucose uptake and suppress hepatic gluconeogenesis, constituting a major risk factor for the metabolic syndrome, type 2 diabetes, and cardiovascular disease (reviewed in Ref. 3). The number of people with type 2 diabetes is expected to increase from 135 million to more than 300 million worldwide by 2025, making it one of the most common chronic diseases.⁴

Shp2 was identified independently by several groups as a Src homology 2 domain-containing non-transmembrane protein Tyr phosphatase.^{5–8} Shp2 is ubiquitously expressed, suggesting a wide range of physiological functions.^{5–8} Mice homozygous for a Shp2 N-terminal deletion mutation die at mid-gestation,^{9,10} prompting Shp2 conditional deletion experiments. In parallel to deletion of Shp2 in postmitotic forebrain neurons of CAMKII α -Cre mice,¹¹ we performed Shp2 ablation in neurons by crossing Shp2 floxed mice with a pan-neuronal Cre-line (CRE3), established and characterized in our laboratory.¹² In this report, we present the “diabetesity” phenotype of the CRE3/Shp2-KO mice and compare it with phenotype exhibited by CAMKII α -Cre:Shp2^{flox/flox} (CaSKO) line.

Materials and Methods

Generation of Mutant Mouse Line with Neuronal Ablation of Shp2

The generation and characterization of the CRE-3 transgenic line were summarized elsewhere.¹² Shp2^{flox} conditional mutant mice (in C57BL/6 background)¹¹ were

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bred for two generations with the transgenic CRE3 mice (FVB/N background). Early generations of obese mice were of mixed genetic background with three fur colors: agouti, black, and white. Thus we backcrossed mice to achieve a complete FVB/N background (white fur color), mating each of nine generations of CRE3/Shp2 heterozygotes with CRE3 homozygous partners. A congenic CRE3/Shp2-KO line was created from N9 heterozygous founders and used for most experimental procedures. Genotyping was undertaken by PCR using primers detecting *Cre* and *Shp2^{fllox}* alleles. A *Cre* 750-bp band was detected by forward and reverse primers (5'-GCCTGCATTACCGGTCGATGCAACGA-3' and 5'-GTGGCAGATGGCGCGCAACACCAT-3', respectively); 450- and 200-bp bands for *Shp2^{fllox}* and wild-type (WT) alleles, respectively, were obtained using forward and reverse primers (5'-ACGTCATGATCCGCTGTCAG-3' and 5'-ATGGGAGGGACAGTGCA GTG-3', respectively). To detect tissue-specific recombination, a pair of *Shp2*-null primers¹¹ was used with the following sequence: forward, 5'-ACGTCATGATCCGCTGTC-3', and reverse, 5'-GCAGGAGACTGCAGCTCAGTGATG-3'.

Measurement of Food Intake, Body Weight, and Temperature

Food Intake

A fixed weight of food was added to cages containing a single animal, and the remaining food was weighed daily. Food intake was measured over 14 days for WT ($n = 12$), heterozygous ($n = 12$), and homozygous ($n = 28$) mice at 8 and 12 weeks of age.

Body Weight

WT ($n = 30$) and CRE3/Shp2-KO ($n = 150$) animals were group-housed and fed standard chow. Body weight was monitored at the same time each week. After 4 weeks of age, animals were weighed monthly.

Acute Thermoregulation after Cold Exposure

Six-month-old CRE3/Shp2-KO mice and WT controls ($n = 8$ per genotype) accustomed to a 25°C environment were exposed singly in empty plastic cages to 4°C for 4 hours without food, and core body temperature was measured at 30-minute intervals. In this paradigm, the ability to maintain core body temperature reflects primarily shivering thermogenesis, with a lesser component of non-shivering thermogenesis.

Analysis of Blood Chemistry, Blood Glucose, Glucose Tolerance, and Serum Insulin and Leptin

The physical status of these mice was closely monitored along with control of the blood biochemistry and neurological examination based on principles of behavioral neuroscience. Mouse sera were collected regularly be-

tween 9:30 and 11:00 AM from the retro-orbital sinus. Overnight fasting (14 hours) and fed glucose levels were measured by a glucometer (Accu-Check Advantage; Roche Diagnostics, Palo Alto, CA) using tail vein blood. Glucose tolerance tests were performed on 1- to 2-, 3- to 6-, and 12-month-old CRE3/Shp2-KO ($n = 6$ to 8 per group) and 3- to 6-month-old WT ($n = 8$) mice fasted for 16 hours. Glucose was injected intraperitoneally at 3 g/kg body weight. Blood samples were collected into EDTA-coated Eppendorf tubes. Glucose levels were measured before and at 15, 30, 60, and 120 minutes after glucose injection. Serum insulin levels were assayed by enzyme-linked immunosorbent assay (Alpco Diagnostic, Salem, NH) using mouse insulin standards (Crystal Chem, Inc., Downers Grove, IL). Values were reported in nanograms per milliliter $\pm$ SEM. Plasma leptin levels were measured by RIA kits from Linco Research (St. Charles, MO). Total serum bilirubin, aspartate aminotransferase, alkaline phosphatase, alanine aminotransferase, triglycerides, cholesterol, high- and low-density lipoproteins, creatinine, blood urea nitrogen, albumin-to-globulin ratio, and basic electrolytes (Na, K, Ca, and P) were determined by the LabCorp Diagnostic Laboratory (San Diego, CA).

X-Ray Examination and Magnetic Resonance Imaging: Measurement of Total Fat Content and Distribution

Faxitron X-ray examination (mx-20 faxitron; Faxitron X-ray Corporation, Wheeling, IL) was performed on living animals to measure skeleton size of age- and sex-matched animals of different genetic backgrounds. Magnetic resonance imaging (MRI) was performed on a 7T MRI machine (General Electric, Milwaukee, WI) equipped with a BFG-200/120-440-S shielded gradient coil. A custom-made frame was used to position the animals anesthetized by inhaled isoflurane (1 to 1.5%). Mice were monitored throughout the experiment using a chest bellows for respiratory rate and a rectal probe for temperature. Air temperature was maintained using a hot air flow system in the magnetic resonance bore.

To acquire body fat images, first a very heavily T1-weighted three-dimensional dataset was produced, which rendered the fat bright and other tissues dark. Applied acquisition parameters were as follows: three-dimensional gradient echo sequence (spoiled grass pulse sequence), echo time 2.1 ms (fat and water in phase), repetition time 5.8, flip angle 12 degrees, bandwidth 83.33, field of view 256 $\times$ 256 $\times$ 50 mm, and matrix 256 $\times$ 256 $\times$ 100 (ie, 0.5-mm isotropic resolution). Using the same parameters, a second dataset was acquired with chemically selective fat saturation enabled, producing a mask image in which fat was dark. Both datasets were coregistered using aligning software (Amira Mercury Computer Systems Solutions, Chelmsford, MA), and the second dataset was subtracted from the first to produce a fat-only image. Images were segmented according to anatomical distribution of body fat (intraperitoneal fat, subcutaneous, and other fat) relative to total animal volume.

In addition, high-resolution MRI investigation was performed on animals that had been found dead and subsequently fixed in Bouin's solution (whole-body fixation) to visualize pathological changes that contributed to mouse mortality. High-resolution imaging parameters were as follows: three-dimensional fast low angle shot (FLASH) pulse sequence, repetition time/echo time 28/6.45 ms, flip angle 25 degrees, field of view 40 mm, and matrix 256 × 3.

Tissue Collection and Analysis

Animals were sacrificed at embryonic day 12, 14.5, 16.5 to 17, postembryonic day 0 to 1, 7, 14, and 1, 3, and 6 months. Embryos were harvested after heart perfusion of mothers with zinc-buffered formalin (Z-fix; Anatech, Inc., Battle Creek, MI); 1- to 14-day-old pups were fixed by imbibition. Older animals were fixed by perfusion, and organs were collected and weighed. After fixation, tissues were processed and embedded in paraffin. In addition to H&E and Masson's trichrome stainings, kidney sections were stained with methenamine silver.

Immunostaining

Fasting 15-week-old female CRE3/Shp2-KO mice and controls received intraperitoneal injections of either saline ($n = 6$ per genotype) or 3 $\mu\text{g/g}$ body weight leptin ($n = 6$ per genotype). At 1 hour after injection, animals were perfused with Z-fix. Brains were sectioned coronally into 3-mm slices, starting from the junction between the hypophysis and chiasma opticum, and processed into paraffin blocks. Dewaxed 5- μm sections were immunostained using an automated immunostainer (Universal Staining System; DakoCytomation, Carpinteria, CA) and using the Envision-Plus-horseradish peroxidase system (DakoCytomation).¹³ Sections were stained as above using antibodies to Shp2 (rabbit polyclonal generated in Dr. G-S Feng's lab¹¹ and two respective monoclonal antibodies from Cell Signaling Technology (Danvers, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), phospho-signal transducer and activator of transcription (STAT) 3 (Ser727), phospho-STAT3 (Tyr705), phospho-extracellular signal-regulated kinase (Erk) 1/2 (all from Cell Signaling), neuropeptide Y (NPY) (Neuromic, Inc., Minneapolis, MN), and pro-opiomelanocortin (Phoenix Pharmaceuticals, Inc., Burlingame, CA).

Immunoblotting

Brain lysates from cortex, hypothalamus, including the arcuate nucleus (ARC), brain stem, or cerebellum derived from control or CRE3/Shp2-KO mice ($n = 5$ per group) were made, and 50 μg of protein per lane was run on SDS-PAGE gels. Immunoblots were blotted with antibodies to Shp2, phospho-STAT3 (Ser727), phospho-STAT3 (Tyr705), and phospho-Erk1/2. HSP60 antibody (Stressgen, Inc., Ann Arbor, MI) served as a loading control. Detection was accomplished using an enhanced chemiluminescence (Amersham Biosciences, Piscat-

away, NJ) multiple antigen detection immunoblotting method, as described previously.¹⁴

Statistical Analysis

Data were analyzed using the STATISTICA software package (StatSoft, Tulsa, OK). Food intake, serum leptin, and insulin levels were analyzed using the one-way analysis of variance test followed by Duncan's post hoc test for multiple comparisons. Mean food intake (grams per day), insulin and leptin levels (nanograms per milliliter) are plotted as a middle point. The box reflects $\pm$ SEM of mean (SEM), and the whiskers mean $\pm 0.95 \times$ SEM. P values < 0.05 were reported as statistically significant. Core body temperature ($^{\circ}\text{C}$), weight (grams), and glucose tolerance tests (milligrams/deciliter) were analyzed in WT and KO mice using unpaired t -tests, with $P < 0.05$ considered a statistically significant difference. The latter was also used to compare leptin serum levels after fasting with those after glucose bolus in the same weight groups of CRE3/Shp2-KO mice, whereby middle points corresponded to mean values and whiskers to mean $\pm 0.95 \times$ SEM.

Results

Neuronal Shp2 Ablation Results in Obesity

We previously established and characterized a transgenic mouse line (CRE3) expressing Cre recombinase specifically in neurons.¹² Neuronal Cre expression was confirmed by virtue of Cre-mediated recombination and activation of a floxed *lacZ* (β -galactosidase) gene in the mouse brain (Figure 1, A and B). Shp2^{fllox} mice¹¹ were mated with the transgenic CRE3 mice. Consecutive breeding of heterozygotes resulted in establishment of a neuron-specific Shp2 KO line (CRE3/Shp2-KO). Homozygosity was confirmed by PCR; all offspring showed Cre and two floxed *Shp2* alleles (Figure 1C, top panel). A homozygous CRE3/Shp2-KO line in an FVB/N background was also established. PCR with *Shp2*-null primers generated a product following Cre-mediated recombination present in brain tissues but not other organs from CRE3/Shp2-KO mice or control (CRE3 or Shp2-floxed) animals, indicating brain-specific recombination (Figure 1C, bottom panel). Using a specific Shp2 antibody,¹¹ Shp2 immunoreactivity was seen in neurons, glia, and the neuropil in the WT, CRE3, and Shp2-floxed control mice, as shown by Shp2 immunostaining in the hypothalamus (ARC and paraventricular nucleus) and hypophysis (Figure 1D, WT). In contrast, loss of Shp2 neuronal staining was observed in CRE3/Shp2-KO mice; a faint Shp2 signal was localized mostly to oligodendroglia (Figure 1D, KO). Immunostaining corroborated immunoblotting results and showed a significant decrease in Shp2 protein levels in the cortex and hypothalamus and less of a reduction in the cerebellum from CRE3/Shp2-KO mice (Figure 1E).

In any genetic background, conditional *Shp2* ablation in neurons produced a significant gain of body weight in

Figure 1. Phenotypic, Faxitron X-ray, and fat-selective MRI analysis of 1-year-old CRE3/Shp2-KO mice. **A** and **B**: In the pan-neuronal CRE line (CRE3), Cre/ β -galactosidase expression was found only in neurons but not in glia [arrows in **B** show satellite glia (red) and astroglia (green) at original magnification $\times 1000$]. **C**: Genotyping and selection of animals was based on PCR analysis using primers that detect a 750-bp Cre band and 450- and 200-bp bands for Shp2^{flx} and wild-type alleles, respectively (top). **C**: A pair of Shp2-null primers show Cre-mediated recombination only in brain, including cortex (CX), hypothalamus (HT), cerebellum (CB), brain stem (BS), and spinal cord (SC), but not in other organs (H, heart; Spl, spleen; and Kid, kidney) from CRE3/Shp2-KO mice (bottom). **D**: Representative Shp2 immunoreactivity in the hypothalamus (ARC) and paraventricular nucleus region from WT and CRE3/Shp2-KO mice. Scale bar represents 500 μ m. **E**: Western blots of cortical, hypothalamic, and cerebellar samples from CRE3, Shp2-flox, and CRE3/Shp2-KO mice were normalized for protein content (50 μ g/lane). A monospecific anti-Shp2 antibody¹¹ detects decreased levels of p63 Shp2 protein in the cortex and hypothalamus from the Shp2-null animals. **F**: Faxitron X-ray examination of living animals shows that CRE3/Shp2-KO and WT mice have similar body lengths, as measured by X-ray images of age- and sex-matched animals with different genetic background (**a**). T1-weighted images of the whole-body MRI of CRE3/Shp2-KO and WT age- and sex-matched controls (**b**). Fat deposits are visualized as an intense white signal used to measure total volume using a computer algorithm.

nearly 50% of CRE3/Shp2-KO mice, starting from ~day 21 after birth. Evaluation of a large mouse colony showed that the overweight phenotype was not strain- or gender-dependent (Figure 1F, a). After 28 months of observation of homozygous CRE3/Shp2-KO animals in a FVB/N background, we found that the maximal body weight gain for the CRE3/Shp2-KO mice was 96 g (487 days old) compared with 44 g for age-matched WT, CRE3, or Shp2-floxed only animals. Necropsy revealed mostly visceral fat deposition, with additional scapular fat pads in a few male animals. Faxitron X-ray examination of living animals showed the same fat distribution pattern and a similar body length of CRE3/Shp2-KO and WT mice, as measured by skeleton size on X-ray images of age- and sex-matched animals (Figure 1F, a). Two groups (five mice per group) of 1-year-old WT and CRE3/Shp2-KO animals were analyzed by MRI to quantitate total fat and white adipose tissue volume (Figure 1F, b) and to inspect internal organs. Using a semimanual segmentation program (Amira Mercury Computer Systems Solutions), visceral and total fat accumulation in CRE3/Shp2-KO mice was found to be two- to threefold that seen in control animals (total volume = 8.03 versus 2.273 ml, respectively; Figure 1F, b). The volume of the brown fat pad remained the same in both groups (data not shown).

CRE3/Shp2-KO Mice Are Hyperphagic

To assess physiological mechanisms leading to weight gain, we measured food intake in individually housed mice starting at 8 and 12 weeks of age for a period of 14 days. A significant difference ($P = 0.0002$) in average daily food intake between homozygous CRE3/Shp2-KO (5.3 ± 0.3 g [3 to 7 g/day]; $n = 28$) and heterozygous with one *Cre* allele (3.7 ± 0.2 g [2.8 to 4.5 g/day]; $n = 12$) or WT mice (3.2 ± 0.2 g [2.5 to 4.0 g/day]; $n = 12$) showed that the homozygotes were hyperphagic (Figure 2A). After approximately 3 months, substantial increases in body weight were observed in CRE3/Shp2-KO mice compared with controls, typically reaching a two-fold gain after 10 months (Figure 2B). After 28 months, 6 of 148 obese mice lost weight spontaneously without showing any physical signs of disease.

Initial weight gain could be attributed not only to hyperphagia but also to alterations in metabolism. As demonstrated by cold exposure analysis (Figure 2C), differences in core body temperature among mice of different genotypes were not statistically significant at the outset of the experiment (WT, 36.4 ± 0.2 ; CRE3/Shp2-KO, 36.3 ± 0.3 ; $P = 0.7$). However, during the course of the experiment, WT mice showed little variation in core body temperature (36.0 ± 0.03), but the temperature of CRE3/Shp2-KO animals dropped precipitously within the 1st hour to 32.3°C and remained at a similar level until the end of the experiment. This result is consistent with an impairment of thermogenesis in CRE3/Shp2-KO animals.

CRE3/Shp2-KO Mice Develop Diabetes

Compared with normoglycemic WT animals, hyperglycemia was detected at 3 months of age in the CRE3/Shp2-KO mice (Figure 2D, a and b). Unlike controls, from then on, CRE3/Shp2-KO mice exhibited no glucose clearance at any time point (up to 120 minutes) after glucose injection, suggesting that neuron-specific *Shp2* deletion causes impaired peripheral glucose disposal (Figure 2D, c and d). However, before reaching 3 months of age, the clearance curve of young CRE3/Shp2-KO mice resembled that of controls (Figure 2D, a and b). All CRE3/Shp2-KO homozygotes heavier than 55 g were hyperinsulinemic with insulin levels proportional to body weight (Figure 2E; $n = 34$). Hyperinsulinemia together with impaired glucose tolerance indicates severe insulin resistance in obese CRE3/Shp2-KO mice.

Neuronal Deletion of Shp2 Leads to Leptin Resistance

Production of leptin, a 16-kDa protein produced primarily in white adipose tissue, is regulated by energy balance (reviewed in Ref. 15). We observed that WT and nonobese, young (<3 months old) CRE3/Shp2-KO adult mice exhibited low serum leptin levels after fasting (data not shown). By contrast, obese CRE3/Shp2-KO animals ($n = 31$) showed hyperleptinemia (~14 to 16 ng/ml) (Figure 2F, a). After a glucose bolus, serum leptin levels dropped in less heavy animals (body weight, 45 to 60 g) but remained high in mice whose body weight exceeded 60 g (Figure 2F, b), suggesting development of leptin resistance.

Insulin and leptin plasma concentrations were also measured in 3-month-old animals 14 hours after fasting and 8 hours after dry chow feeding ad libitum (Figure 2, G–J). During fasting, WT and heterozygous CRE3/Shp2-KO animals maintained normal insulin levels of 0.1 to 0.6 ng/dl (mean, 0.5 ± 0.1 ng/dl; $n = 12$ for both genotypes), whereas homozygous CRE3/Shp2 nulls showed a significantly higher average level of insulin (1.2 ± 0.2 ; $n = 12$) ($P < 0.0001$; Figure 2G). After dry food consumption, insulin values in WT mice (mean, 1.5 ± 0.2 ng/dl) were almost identical to those seen in fasting CRE3/Shp2-KO animals (1.2 to 1.5 ng/dl) (Figure 2, G and H). A gradual increase in insulin serum levels was observed in heterozygous and homozygous CRE3/Shp2-KO mice, reaching the highest values in the latter group (mean, 6.0 ± 0.9 mg/ml) (Figure 2H; $P = 0.0002$; $n = 12$ for each genotype). Feeding elevated average leptin levels from 0.8 ± 0.1 to 3.7 ± 0.5 ng/ml in WT animals and from 8.9 ± 1.1 to 15.2 ± 0.4 ng/ml in the heterozygous CRE3/Shp2 mice (Figure 2, I and J). However, leptin blood values remained unchanged and were approximately at the same high levels in homozygous CRE3/Shp2-KO mice both without (14.3 ± 0.8 ng/ml) or after food consumption (14.2 ± 0.8 ng/ml) (Figure 2, I and J).

Thus, preliminary analysis of the CRE3/Shp2-KO line indicated a phenotype characterized by hyperphagia,

Figure 2. CRE3/Shp2-KO mice are hyperphagic, obese, hyperglycemic, hyperinsulinemic, and hyperleptinemic. **A:** Food intake was measured (g/day) in WT ($n = 12$), heterozygous (HT; $n = 12$), and homozygous (HO; $n = 28$) animals. **B:** Body weights of CRE3/Shp2-KO ($n = 150$) and WT ($n = 30$) mice were measured at the indicated time points. **C:** Core body temperature was measured in WT and CRE3/Shp2-KO mice ($n = 8$ per genotype) before and at 30-minute intervals after cold exposure (4°C). **D:** Glucose tolerance tests were performed on CRE3/Shp2-KO mice at 1 to 2 months (**b**), 3 to 6 months (**c**), and 12 months (**d**) ($n = 6$ to 8 per group) and on 3- to 6-month-old WT mice (**a**; $n = 8$) at indicated time points after 16 hours of fasting. $*P < 0.05$; $**P < 0.001$ compared with WT. **E:** Insulin serum levels in fasted CRE3/Shp2-KO mice ($n = 34$) were correlated with body weight. **F:** Leptin serum levels measured in CRE3/Shp2-KO mice ($n = 31$) after overnight fasting (**a**) and after a glucose bolus (3 g/body weight) (**b**) were correlated with body weight ($**P < 0.001$). **G–J:** Serum insulin and leptin measurements were performed in fasted (**G** and **I**) or ad libitum-fed (**H** and **J**) WT, HT, and HO 3-month-old animals ($n = 12$ per genotype). Mean values are plotted as a marker; whiskers reflect ± 1.96 SEM from the mean.

obesity, hyperglycemia, hyperinsulinemia, hyperleptinemia, and insulin and leptin resistance. Obese mice developed diabetes at approximately 3 to 5 months of age, whereas heterozygous CRE3/Shp2 animals maintained normal blood glucose and weight throughout the entire 28-month observation.

Development of Morbid Obesity and Related Pathology

During 28 months of observation, 148 homozygous CRE3/Shp2-KO, 54 heterozygotes, 45 WT FVB/N CRE3, and 25 C57BL/6 SHP2-floxed control mice were monitored. Whereas only 4 controls died during the period, 32 obese CRE3/Shp2 homozygotes died, and 45 (total 52%) were sacrificed because of symptoms of physical sickness, eg, fur appearance, somnolence, and decreased mobility. Other than sporadic seizure-like tremor noted in

two CRE3/Shp2-KO mice, no neurological symptoms were observed in sick animals. MRI analysis of a randomly selected 14-month-old obese CRE3/Shp2-KO mouse (Figure 3A) demonstrated white adipose tissue accumulation (Figure 3B; red arrows), enlarged kidneys with a thin rim of cortex, and dilated collecting system and renal pelvis (Figure 3B; green arrows) compared with a WT animal (Figure 3C). Necropsy and histological examination confirmed renal pathological changes (Figure 3, D–I). In 26 of 45 obese, sick-appearing animals, renal alterations included subacute or chronic pyelonephritis with granulocyte/lymphocytic infiltrates obliterating the lumen of the renal pelvis and surrounding collecting ducts, vasculitis, glomerulonephritis, and occasional cysts, leaving a very thin rim of functioning renal cortex (Figure 3, D–I; data not shown). The occurrence of cystic and hemorrhagic damage may be related to thromboembolism or ischemic infarcts. Glomerular changes related

Figure 3. Representative pathological changes in the obese CRE3/Shp2-KO mice. MRI analysis (**B** and **C**) of a morbidly obese CRE3/Shp2-KO mouse (**A** and **B**) and a WT counterpart (**C**); **red arrows** point to body fat, **green arrows** point to kidneys. Gross (**D**) and microscopic (**E–I**) examination of kidney of this case [original magnification: $\times 60$ (**E**), $\times 1000$ (**inset** in **E**), $\times 200$ (**F–H**), and $\times 400$ (**I**)]. **J–M**: Spectrum of renal pathology related to diabetic nephropathy and, respectively, the mesangial injury (**J**; H&E; original magnification $\times 250$) and thickening of glomerular basement membrane (**K**; original magnification $\times 250$) in Jones's silver staining (**L** and **M**; original magnification $\times 400$) and Kimmelstiel-Wilson-like nodules (**L**) and progressive glomerulosclerosis (**M**) in Masson-Trichrome staining. **N–V**: male with gastric and bladder paresis and, respectively, stomach (**N** in gross and **O** in microscopic original magnification $\times 15$), urinary bladder (**N** in gross and **P** in original magnification $\times 10$), prostate with prostatitis [original magnification: $\times 15$ (**Q**), $\times 800$ (**inset** in **Q**), and $\times 200$ (**R** and **S**)], vas deferens with inflammation [original magnification: $\times 30$ (**T**) and $\times 60$ (**inset**)], seminiferous tubules with paucity of germ cells (original magnification, $\times 300$ in **U**) and epididymis with aspermia (original magnification, $\times 150$ in **V**). Immunostaining reveals that inflammatory infiltrates in kidney and prostate consist of B220-positive B cells (**G** and **R**) and Ly6G-positive granulocytes (**H**, **I**, and **S**).

to developing diabetic nephropathy were first observed after 5 to 6 months of age and became more pronounced after 20 months in mice with persistently elevated blood glucose levels. The glomerular alterations included mesangial injury and diffuse matrix expansion with insudation of plasma proteins and moderate thickening of glomerular basement membrane (Figure 3, J and K). During disease progression, nodular sclerosis resembling Kimmel-Steel-Wilson lesions in humans (Figure 3L), hyalinization, and PAS-positive deposits in areas of mesangiolysis, fibrosis, and glomerulosclerosis (Figure 3M) has been observed in 17 of 77 (22%) of terminally sick animals. In addition, tubulointerstitial alterations, such as expanded renal medullary interstitium, vacuolization of tubular cells, tubular dilatation, and interstitial fibrosis with increased number of interstitial leukocytes, lymphocytes, and macrophages were noted.

Nine of 77 (12%) terminally sick CRE3/Shp2-KO mice showed an extremely extended stomach (Figure 3, N and O) and bladder (Figure 3, N and P). In the former, the stomach wall was thin and flattened and largely replaced by squamous epithelium (Figure 3O). In the hypertrophic bladder, total wall mass was nearly doubled primarily due to increased smooth muscle. Numerous lymphoid aggregates in the lamina propria of the urinary bladder were seen accompanied by infiltration of predominantly mononuclear cells in the bladder wall, suggestive of chronic inflammation. Increased urinary output and frequency were observed. In obese males (6 of 29 [20%]), renal inflammatory changes were accompanied by florid bacterial infection of the urinary bladder, prostate (Figure 3, P–S), seminal vesicles, or adipose tissue surrounding the vas deferens (Figure 3T). At 4 to 6 months of age, obese homozygotes could not reproduce. Histological examination revealed testicular degeneration with reduced numbers of germinal epithelial cells. In more advanced degeneration of seminiferous tubules, only Sertoli cells and occasional spermatogonia remained (Figure 3U); no sperm were visible in the epididymis (Figure 3V).

A major cause of tissue damage in poorly controlled diabetes is vascular disease affecting both the micro- and macrovasculature. Vasculitis characterized by lymphocytic and granulocytic inflammatory infiltrates and thickened tunica media was observed in numerous organs, including kidney (Figure 4A) and heart (Figure 4B).

We also examined pancreatic tissue from obese CRE3/Shp2-KO mice. Young adult animals (3 to 6 months old) exhibited islet β -cell hyperplasia (Figure 4, C and D). Morphometric analysis of β -cell mass at 3 months of age showed a two- to fourfold increase in the CRE3/Shp2-KO group and about a ninefold increase at 6 months (data not shown), compared with controls, indicating a compensatory response of islets to insulin resistance. Twenty-four of 30 (80%) obese mice investigated at 6, 12, 16, 21, and 28 months, revealed the same compensatory islet phenotype. To determine whether islet hyperplasia was due to an increase in β -cell versus non- β -cell types, we costained pancreas tissue for expression of key markers of islet function, including insulin, somatostatin, and glucagon. As expected, CRE3/Shp2-KO mice showed an approximately threefold increase in islet size due to an

increase in β -cell mass, whereas non- β cells were scattered throughout the islets (Figure 4D). In addition, lymphocyte infiltrates surrounding islets (Figure 4E), fibrotic changes in pancreatic ducts, and hypertrophy of media in pancreatic arteries (Figure 4F) were observed in several CRE3/Shp2-KO animals. Abundant inflammatory cell infiltrates were localized in adipose tissue in a proportion of CRE3/Shp2-KO mice (Figure 4G), suggestive of tertiary lymphoid organ neogenesis.

Older, obese CRE3/Shp2-KO mice displayed morphological characteristics of fatty liver (Figure 4H); however, serum liver enzymes (aspartate aminotransferase and alanine aminotransferase) and total serum bilirubin remained within the normal range (Table 1).

The results of sera biochemical analysis of basic electrolytes, metabolic parameters, and renal function analyses are summarized in Table 1. These data are consistent with renal pathology exhibited by CRE3/Shp2-KO mice: elevated blood urea nitrogen, $P = 0.008$; creatinine, $P = 0.006$; decreased albumin-to-globulin ratio, $P < 0.0001$; metabolic disturbances with elevated phosphorus, $P = 0.02$; low levels of calcium, $P = 0.02$; high glucose and pro-atherogenic lipoproteins profile (high cholesterol, $P = 0.02$; triglyceride, $P = 0.04$; and low-density lipoprotein, $P = 0.002$ levels) (Table 1; Figure 5).

The Immediate Leptin Responses Are Altered in the Hypothalamic Neurons

To examine the cellular responsive signals immediately downstream of the leptin receptor in the hypothalamus, immunohistochemistry was undertaken on serial paraffin sections (Figure 6, A–Y). Unlike immunoblotting, the immunohistochemistry approach allows discrimination between protein expressed in neuronal versus glial cells. Administration of a leptin bolus slightly decreased expression of NPY in ARC neurons of WT controls and CRE3/Shp2-KO animals, but expression levels between the two groups did not differ significantly (Figure 6, B, D, F, and H). In contrast, knockout mice responded to leptin administration with a striking increase in the NPY signal in other brain regions, such as the hippocampus, amygdaloid nucleus, olfactory bulb, and perifornical area of the entorhinal and in neo-cortex (Figure 6, A–I). In hypothalamus, NPY was expressed predominantly periventricularly in the dorso- and ventromedial hypothalamic and the adjacent arcuate nuclei in the WT animals (Figure 6, B and D) and mostly in the nuclei of lateral hypothalamus in the CRE3/Shp2-KO mice (Figure 6, F, H, and I). During fasting, a low POMC signal was detected in ARC neurons of WT controls (Figure 6J); that signal increased considerably after leptin injection (Figure 6K). Surprisingly, CRE3/Shp2-KO mice demonstrated elevated basal levels of neuronal POMC before the leptin bolus (Figure 6L; $P < 0.0001$). The intensity and prevalence of that signal was comparable with that observed in WT animals after leptin injection. After a leptin bolus, CRE3/Shp2-KO mice maintained elevated POMC levels (Figure 6M).

Leptin injection increased phosphorylation of STAT3 Tyr705 in WT ARC neurons (Figure 6, N–Q). In contrast,

Figure 4. Pathology of blood vessels, pancreas, and liver in CRE3/Shp2-KO mice. Histological examination revealed vasculitis (**A**, **B**, and **F**) in several organs, including kidney (**A**), heart (**B**), and pancreas (**F**), compensatory islet β -cell hyperplasia (**C** and **D**; **D**, double-staining for insulin [blue] and glucagon [red]), lymphocyte infiltrations of pancreatic islets (**E**), and adipose tissue (**G**). **H**: Fatty liver morphology in a CRE3/Shp2-KO mouse. Original magnification: $\times 250$ (**A**); $\times 40$ (**B**); $\times 100$ (inset in **B**); $\times 60$ (**C** and **D**); $\times 200$ (**E**, **G**, and **H**); and $\times 60$ (**F**).

Table 1. Serum Biochemical Analysis of Basic Electrolytes and Metabolic and Renal Function

Serum biochemistry	WT (n = 12)	CRE3/Shp2-KO (n = 16)	P value
AST (IU/L)	206.4 ± 32.7	276.9 ± 48.7	0.3
ALT	57.8 ± 13.7	44.4 ± 10.8	0.4
Total bilirubin (mg/dL)	0.2 ± 0.02	0.3 ± 0.05	0.2
BUN (mg/dL)	23.1 ± 1.08	46.7 ± 7.07	0.008
Creatinine (mg/dL)	0.06 ± 0.01	1.3 ± 0.37	0.006
A/G	2.1 ± 0.1	1.3 ± 0.1	<0.0001
Calcium (mg/dL)	9.0 ± 0.2	7.9 ± 0.3	0.02
Phosphorus (mg/dL)	6.5 ± 0.4	21.4 ± 5.1	0.02
Triglycerides (mg/dL)	86.1 ± 8.0	130.3 ± 16.3	0.04
Cholesterol (mg/dL)	127.2 ± 10.6	170.4 ± 12.1	0.02
HDL (mg/dL)	94.5 ± 8.5	81.3 ± 10.5	0.4
LDL (mg/dL)	25.7 ± 2.9	51.1 ± 5.9	0.002

A/G, albumin/globulin ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

obese CRE3/Shp2-KO mice showed high basal levels of phospho-Tyr STAT3 (Figure 6P; $P = 0.002$), and that signal was decreased rather than induced after leptin injection (Figure 6Q; $P = 0.0002$). Similarly, induction of STAT3 Ser727 phosphorylation was observed in WT ARC neurons after a leptin bolus (Figure 6, R and S; $P =$

0.0009), whereas CRE3/Shp2-KO mice showed a high basal signal that slightly decreased on leptin treatment (Figure 6, T and U; $P = 0.01$). Consistent with these observations, phospho-ERK1/2 expression in CRE3/Shp2-KO animals during fasting was higher than that seen in WT mice (Figure 6, V and X; $P = 0.0002$) and decreased after the leptin bolus (Figure 6Y; $P = 0.0001$). In ARC neurons of WT animals, an insignificant increase in ERK1/2 activation (Thr218/Tyr220) in response to leptin injection was seen (Figure 6W).

Discussion

In this study, we characterized functional consequences of neuron-specific Shp2 deletion. This mutation in CRE3/Shp2-KO mice had significant effects on food intake and body weight, leading to obesity with associated pathophysiological complications, such as hyperglycemia, hyperinsulinemia, hyperleptinemia, vasculitis, nephropathy, urinary bladder infections, prostatitis, gastric paresis, and impaired spermatogenesis. During 28 months of observation, approximately one-half of homozygous, obese CRE3/Shp2-KO mice suffered or died from complications of diabetes that resemble those encountered in humans.

Currently, the main therapeutic problem in diabetes is not management of its acute metabolic disturbances but prevention and treatment of its chronic complications, which encompass nearly all organ systems. Although a large number of animal models have been produced to dissect the genetics of diabetes and/or obesity, it is disputed whether any currently available animal model precisely reflects the diabetic complications in humans (reviewed in Ref. 16). The developing insulin and leptin resistance leads to vasculopathy and clinical complications, including diabetic neuropathy. Similar to human disease, CRE3/Shp2-KO mice demonstrate upper gastrointestinal symptoms attributable to delayed emptying of the stomach due to autonomic neuropathy, hypomotility, and gastroparesis,¹⁷ as well as urinary bladder dysfunction, one of the most common diabetic complications, which is associated with increased urinary output

Figure 5. Blood biochemistry in WT and CRE3/Shp2-KO mice. Serum blood urea nitrogen (BUN), creatinine, albumin-to-globulin ratio (A/G), basic electrolytes such as phosphorus, low-density lipoprotein, and triglyceride levels were significantly altered in CRE3/Shp2-KO mice compared with WT.

and frequency, bladder paralysis and urinary tract infections.¹⁸ Glomerular changes in this experimental model closely resemble those seen in human diabetic nephropathy. Obesity and diabetes are predisposing factors for nonalcoholic fatty liver, a precursor of steatohepatitis, both in human insulin resistance and in our experimental model, where the liver is programmed for lipogenesis rather than for glycogenesis. Thus, the pathology observed in our CRE3/Shp2-KO model resembles human diabetes.

Shp2 expression occurs throughout mouse brain development in proliferating neuronal and glial progenitors as well as in postmitotic cells.¹⁹ Neuronal Shp2 immunoreactivity, not restricted to a specific neuronal type or location, has been detected in the adult human nervous system.²⁰ In addition to neuronal Shp2 immunostaining, we observed high levels of Shp2 expression in oligodendroglial cells in naive mice. Increased susceptibility to ischemia-induced brain damage observed in transgenic mice overexpressing a dominant-negative form of Shp2 indicated Shp2 involvement in neuronal survival pathways.²¹ In this study, neuronal ablation of Shp2 did not produce obvious developmental brain malformations despite the fact that Shp2 excision occurred at approximately gestational (embryonic) day 11.5 to 12 in cells committed to a neuronal (rather than glial) fate and in differentiating neurons.

Previous studies demonstrated Shp2 regulation of Erk activation in response to a variety of growth factors and cytokines.^{22,23} Typically, stimulation of the leptin receptor engages the Tyr kinase Janus tyrosine kinase 2, which in turn phosphorylates Tyr1138 and Tyr985 on the leptin receptor.²⁴ Although Tyr1138 is required for docking of the transcription factor STAT3, previous reports show recruitment of Shp2 to Tyr985 of the leptin receptor, which in turn activates the canonical Ras-Erk pathway. Leptin resistance has been shown to be mediated by negative regulators of leptin signaling, protein-Tyr phosphatase 1B^{25–27} and the suppressors of cytokine signaling family,²⁸ such as suppressors of cytokine signaling 3.²⁹ Suppressors of cytokine signaling 3 can compete with Shp2 for Tyr985 binding, inhibiting leptin signaling.³⁰ A knockin mutant mouse harboring a Y985L mutation was recently reported, attempting to address the role of Tyr985 in leptin signaling.³¹ However, the phenotype is confounded by the replacement of Tyr with leucine, which may distort the conformation of the receptor molecule; characterization of a Y985F mutant will certainly help clarify the issue. A similar concern exists for the Y1138S mutant,³² because the introduced Ser residue may become an artificial kinase target thereby producing an artificial phenotype, which could be partially clarified by characterizing a Y1138F knockin mutant. After a leptin bolus, we observed phosphorylation of STAT3 Tyr705, implicating involvement of Janus tyrosine kinase 2.³³ The phosphorylation event changes protein conformation and enables homodimerization, nuclear translocation, and DNA binding of STAT3 to leptin targets.³⁴ However, DNA binding is not sufficient for STAT3 transcriptional activation, and additional phosphorylation of Ser727 is required to recruit the p300 transcriptional coactivator.³⁵ The phosphorylation consensus includes the requisite serine-

rich phosphorylation motif for mitogen-activated protein kinases.³⁶ Thus Shp2-dependent activation and nuclear translocation of Erk kinase serves as a second key event activating STAT3 transcriptional activity.^{37–39} Effects of leptin on feeding behavior are mediated by its downstream targets, including the activation of anorectic peptides derived from the POMC gene and repression of genes encoding orexigenic NPY and Agouti-related protein peptides. However, CRE3/Shp2-KO mice responded to leptin stimulation with a striking increase in the NPY signal in selected brain regions, implicating dysregulation of the central appetite control. As expected, fasting WT mice responded to a leptin bolus by up-regulating STAT3 Tyr and Ser phosphorylation and an increase in POMC protein levels, a known STAT3 target. Basal Tyr and Ser phosphorylation of STAT3 was enhanced in CRE3/Shp2-KO mice compared with controls and was significantly (Tyr) and modestly (Ser) down-regulated after leptin treatment. Both insulin and leptin pathways converge at the level of the insulin receptor substrate-phosphatidylinositol 3-kinase pathway,⁴⁰ and further investigation is required to elucidate the role of Shp2 in insulin and leptin signaling.

Although the CRE3/Shp2-KO model recapitulates some aspects of the CaSKO phenotype,¹¹ it also brings forth striking differences. In the CaSKO model, Shp2 was selectively deleted in postmitotic forebrain neurons, whereas the CRE3/Shp2-KO mice exhibit pan-neuronal Shp2 ablation. Although an obese phenotype is seen in both conditional models, they differ significantly at metabolic and signaling levels. Whereas appetite deregulation leads to several pathological conditions in CRE3/Shp2-KO line, CaSKO mice are normophagic. Unlike the CaSKO line, which exhibits increased linear growth, the CRE3/SHP2-KO mice are normal in length. Although both lines are hyperinsulinemic, in contrast to the CRE3/Shp2-KO line, CaSKO mice become hypoglycemic after fasting. In terms of signaling, CaSKO mice show no difference in POMC expression levels after fasting and demonstrate reduced Erk phosphorylation.¹¹ CRE3/Shp2-KO mice, however, exhibit high basal phosphorylation of phospho-Tyr, Ser-STAT3, and phospho-Erk in ARC neurons. These results indicate that in our mouse model, the receptor long form of the leptin receptor (LRb) activity and STAT3 signaling are intact, presumably activated by high leptin levels in the obese line. Activation of the canonical LRb-STAT3 pathway could be responsible for the observed high POMC levels; however, it seems to be ineffective for appetite regulation. A question arises as to why high POMC fails to decrease NPY levels and reduce appetite. As expected, a leptin bolus down-regulated NPY in ARC neurons but paradoxically up-regulated NPY in centers regulating autonomic functions, such as the hippocampus and collaterals of the olfactory system. Further brain analysis of our CRE3/Shp2-KO line may elucidate molecular mechanisms of appetite deregulation leading to obesity and diabetes.

In summary, neuron-specific Shp2 deletion in CRE3-Shp2^{lox/lox} mice leads to obesity and diabetes with associated pathophysiological complications resembling human pathology. The proposed mouse model may help to

elucidate molecular mechanisms underlying human diabetes. Further research should be conducted to determine the role of Shp2 in insulin and leptin signaling.

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