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ORIGINAL ARTICLE

Lack of the serotonin transporter in mice reduces locomotor activity and leads to gender-dependent late onset obesity

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Objective: Mice deficient of the serotonin transporter (5-HTT ko) mice have a reduced brain serotonin content and develop late-onset obesity. To elucidate the pathophysiology of this obesity, we analyzed the expression of the interrelated weight-regulatory molecules: brain-derived neurotrophic factor (BDNF) and leptin receptor (LR) in brain areas associated with nutrition and activity.
Research Design and Methods: We investigated feeding behavior, physical activity and metabolic parameters of 5-HTT ko and wild-type mice and measured the expression of BDNF and LR in brain areas associated with nutrition and activity using quantitative real-time PCR. The influence of age, gender and fasting was analyzed.
Results: Male 5-HTT ko mice developed obesity without hyperphagia from the age of 5 months. Physical activity was reduced in old male, but not old female, 5-HTT ko mice. The BDNF gene expression in frontal cortex was elevated in young, but reduced in old 5-HTT ko mice. Fasting failed to increase the BDNF gene expression in frontal cortex of young 5 HTT ko mice and in the hypothalamus in old 5-HTT ko mice. The fasting-induced hypothalamic increase of LR was absent in both young and old 5-HTT ko mice.
Conclusions: We propose that low brain serotonin level due to the 5-HTT ko genotype leads to reduced physical activity and low BDNF, which together with the lack of fasting-induced hypothalamic BDNF and LR production results in late-onset obesity. Although lack of the 5-HTT is a genetic vulnerability factor for obesity, female gender is protective.
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Keywords: brain-derived neurotrophic factor (BDNF); leptin receptor; serotonin transporter; locomotor activity; feeding behavior

Introduction

Obesity is a growing health-care problem worldwide. It has considerable associated morbidity and mortality with increased rates of coronary heart disease, diabetes, hypertension and cancer. Besides food intake, physical activity is an important determinant of body weight.[1] The molecular mediators linking the central nervous and endocrine system in the regulation of energy balance include adipocytokines, such as leptin or adiponectin,[2,3] pro-inflammatory cytokines[4] and serotonin (5-hydroxytryptamine, 5-HT),[5] and neurotrophins such as brain-derived neurotrophic factor (BDNF).[6] To study the central regulation of food intake and body activity, mouse models of obesity have been designed and characterized, including models for early- and late-onset obesity.[7] In our colony of serotonin transporter knockout (5-HTT ko) mice, we observed that these mice become obese with age. Given that 5-HT is important for feeding behavior and metabolism[8] and acts as an anorexigenic factor,[9] it is conceivable that reduced brain 5-HT levels in 5-HTT ko mice contribute to their obesity.[10] However, cerebral 5-HT levels are reduced in these mice already at an early age, when body weight is still normal.[11] We therefore aimed at identifying additional mediators in the central regulation of body weight in 5-HTT ko mice.

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The neurotrophin BDNF is highly expressed in the ventromedial hypothalamus. It improves lipid and glucose utilization and controls energy homeostasis and feeding behavior.[12] Serotonergic neurons express the main BDNF receptor tyrosine kinase B (TrkB),[13] and 5-HT and BDNF co-regulate each other.[14] Brain-derived neurotrophic factor supports the maintenance of serotonergic neurons and the secretion of 5-HT. Similarily, 5-HT induces the expression and increases the secretion level of BDNF. Furthermore, endogenous BDNF influences the consequences of 5-HTT deficiency in a gender-dependent manner, such that female 5-HTT ko mice are less vulnerable to the lack of BDNF.[15] Serotonergic neurons also express leptin receptors (LRs), through which the adipocytokine leptin inhibits feeding behavior.[16] We therefore set out to investigate the interrelation of 5-HT, BDNF and LR in 5-HTT ko mice and report on a new model of adult-onset obesity.

Materials and methods

Animals

We investigated 82 5-HTT ko mice (male/female ratio = 43/39) and 86 wild-type (WT) littermates of C57BL/6 background (male/female ratio = 45/41). The 5-HTT ko mice were generated as described earlier.[11] All mice were held under standard conditions with a 12:12-h light:dark cycle and at an average room temperature of 22–24 °C. Two age groups were examined: 38 young 5-HTT ko mice (mean age: 3.5 ± 0.01 months) and 49 young WT mice (mean age: 3.6 ± 0.01 months), and 44 old 5-HTT ko mice (mean age: 15.3 ± 2.1 months) and 37 old WT mice (mean age: 14.5 ± 2.1 months). Mice were fed standard chow (commercially prepared complete diet) and had access to water *ad libitum*, except during the period of the fasting experiment (see below). All experiments were approved by the Bavarian State authorities.

Behavioral testing and measurement of vital parameters

Physical activity was monitored on a treadmill (Tecniplast, Hohenpreißenberg, Germany) and evaluated as rounds per day over a period of 6 days. The treadmill was not provided in the home cage during non-testing times. During the testing period, mice were restricted to the treadmill. The treadmill was moved by the mice, no fixed velocity was set. Food ingestion was measured by individually weighing the fodder in the morning and evening of the test day. Body weight was measured before and after treadmill exercise. Skin temperature was measured every morning during the six days of experiment using a digital infrared thermometer (Raytek, Berlin, Germany).

Fasting experiments

Before blood withdrawal and tissue collection, each age group was divided into two subgroups consisting of fasted and normally fed animals. While the normally fed animals had food and water access *ad libitum*, fasted ones underwent food deprivation for 12 h (1900–0700 hours).

Blood withdrawal and analysis

Whole blood was withdrawn when mice were under deep pentobarbital anesthesia (Narcoren, 0.2 mg kg^{-1} body weight) from the axillary vessels. Serum samples (3300 g; 10 min; 4 °C) were analyzed for leptin (mouse leptin RIA kit; Linco, St Charles, MO, USA), insulin (mouse insulin ELISA kit; Chrystal Chem, Chicago, IL, USA), adiponectin (mouse adiponectin ELISA kit; Linco) and glucose (Beckman Glucose Analyzer II, Beckman Instruments, Fullerton, CA, USA) using commercial radio and enzyme-linked immunosorbent assays. Blood withdrawal was always performed in the first hour after 12 h of overnight fasting in all animals (that is, 0800 hours).

Tissue collection

For quantitative RT–PCR analysis, brain areas were dissected after decapitation and exsanguination when mice were under deep pentobarbital anesthesia. Frontal cortex, hypothalamus and thalamus were bilaterally exposed and dissected carefully (according to www.mbl.org). Samples were shock-frozen in liquid nitrogen and stored at −80 °C before further processing. For oil-red staining, a 2–3 mm^2 piece of liver was dissected for investigation of fat storage before and after fasting. Samples were embedded in OCT medium (optimal cutting temperature, TissueTek, Sakura, Berlin, Germany, immediately after excorporation, shock-frozen in 2-methyl butane pre-cooled in liquid nitrogen and stored at −80 °C. For the investigation of hypothalamic nuclei with antibodies against BDNF and LR, the complete mouse brain was extracted after decapitation under deep barbiturate narcosis. After removing of the cerebellum, the entire brain was embedded in TissueTek and shock-frozen in a 2-methyl butane-containing cup placed in liquid nitrogen and was stored at −80 °C.

RNA extraction

The extraction of RNA was performed by following a modified version of the protocol of Chomczynski and Sacchi.[17] In brief, the frozen tissue samples were thawed on ice and incubated with TRIzol reagent (Invitrogen, Karlsruhe, Germany). After homogenization (Ultraturrax homogenizer Polytron PT 1600E, Kinematica, Luzern, Switzerland) and addition of chloroform, samples were centrifuged (13 000 *g*; 4 °C; 15 min). The upper phase was mixed with glycogen and propanol, followed by overnight incubation at −20 °C, washing with 75% ethanol and dissolving in diethylpyrocarbonate-treated water. The messenger RNA (mRNA) yield was quantified photometrically (Eppendorf, Hamburg, Germany) and the 260/280 ratio was measured for the integrity of the extracted RNA.

Reverse transcription PCR
TaqMan Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany) were used. The reaction contained 500 ng of mRNA, 10 × reaction buffer, dNTPs, $MgCl_2$, random hexameres, RNAse inhibitor and multiscribe reverse transcriptase. The PCR reactions were run at a final volume of 100 µl using the 96-well GeneAmp PCR System 9700 cycler (Applied Biosystems). The following conditions were applied: 10 min at 38 °C, 60 min at 48 °C and 25 min at 95 °C.

Quantitative RT–PCR
All quantitative RT–PCR reactions were run using the GeneAmp 7700 sequence detection system (Applied Biosystems). Reactions contained 5 µl of complementary DNA and TaqMan Universal Master Mix (Applied Biosystems). Gene-specific oligonucleotide primers and probes for murine BDNF (Assay-ID: Mm01334042_m1) and LR (Assay-ID: Mm00440181_m1), and for the endogenous control 18S RNA were obtained as TaqMan Gene Expression Assays (Applied Biosystems). The reactions contained 25 µl TaqMan Universal Master Mix and 2.5 µl of the specific primer adjusted to a final volume of 50 µl with water. The cycler conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C (40 cycles), 1 min at 60 °C. Each quantitative RT-PCR plate contained a tissue- and primer-specific calibrator sample, which was the sample from an untreated mouse, threshold cycles values for which (C_t values) were next to the calculated mean of all control samples. The absolute value of the calibrator was set as 1 and all measured samples were related to this sample. To guarantee primer specificity and to exclude genomic contamination, negative controls without complementary DNA template were run on each real-time PCR well plate. All samples were measured as triplicates. Data were evaluated using the comparative $\Delta\Delta C_t$ method, as described by the manufacturer (see User bulletin #2 Applied Biosystems, P/N 4303859, 1997). In brief, the C_t values of the measured target are normalized to the endogenous control and the values are related to the calibrator. This relation is given by $2_t^{-\Delta\Delta C}$.

Immunohistochemistry and oil-red staining
For immunohistochemistry, 10-µm cryosections of the extracted entire mouse brain were cut using a cryostat (Leica, Bensheim, Germany). The ventromedial hypothalamus was localized by hemalaun–eosin staining. The immunohistochemical staining with antibodies against BDNF (1:100; Chemicon, Temecula, CA, USA) and LR (1:500; ABR, Hamburg, Germany) was performed following standard methods, as previously described,[18] using an avidine–biotine system (Vector Laboratories, Burlingame, CA, USA), 0.02% diaminobenzidine as chromogen and hemalaun as a counter stain. For each animal, two sections were analyzed for presence of BDNF- or LR-positive cells.

For oil-red staining, 10-µm liver sections were stained for morphometric analysis with a Zeiss Axiophot 2 microscope (Oberkochen, Germany). Further analysis was performed using Image Pro Plus software (Version 4.0, Media Cybernetics, Silver Spring, MD, USA). The proportion of fat was determined by the investigation of three randomly selected 3 × 3 µm areas of each section.

Statistical analysis and graphs
The SPSS software (Version 16.0.2; Munich, Germany) was used for graphs and calculations. One-way analysis of variance with correction for multiple testing was used for behavioral and metabolic data (means ± s.e.m.); for all other data, the Mann–Whitney *U* test was used for group comparison (median and range). The *P*-values <0.05 were considered statistically significant.

Results

Male 5-HTT ko mice develop late-onset obesity, are physically less active than WT mice, but do not differ in feeding behavior
Body weight was not different between young (that is, <5 months) male and female 5-HTT ko and WT mice. Older male 5-HTT ko mice had markedly higher body weight than male WT mice starting at the age of 5 months ($P<0.0001$; Figures 1a–c). This was not associated with an increase in body length (data not shown). In the treadmill test, young 5-HTT ko mice of both genders (mean age: 3.5 ± 0.01 months) were physically less active than WT mice (female: $P<0.001$; male: $P=0.001$; Figure 1d). In the group of old mice (mean age: 15 ± 2 months), male 5-HTT ko mice were less active than male WT mice ($P=0.002$) female 5-HTT ko mice ($P=0.001$; Figure 1d). Old female 5-HTT ko mice had the same level of activity as WT mice of the same age.

Young mice of both genotypes lost weight equally after 6 days in the treadmill (Figure 1e). Although old WT mice and old female 5-HTT ko mice also lost weight after 6 days in the treadmill, old male 5-HTT ko mice did not, ($P=0.02$; Figure 1e). Food intake did not differ between 5-HTT ko and WT mice of any age and gender (Figure 1f). Body temperature was not different between genotypes and genders in young and old mice (data not shown).

Age- and tissue-specific regulation of BDNF and LR under sated and fasting conditions
Leptin and BDNF are regulators of metabolism and interact with the serotonergic system. As BDNF and LR expressions increase after fasting,[19,20] we investigated BDNF and LR gene expression in brain tissue from sated and fasted mice. Brain areas associated with feeding behavior and the regulation of physical activity were chosen for analysis. In the frontal cortex of sated young 5-HTT ko mice, BDNF gene expression was higher than that in young WT mice ($P=0.03$; Figure 2a).

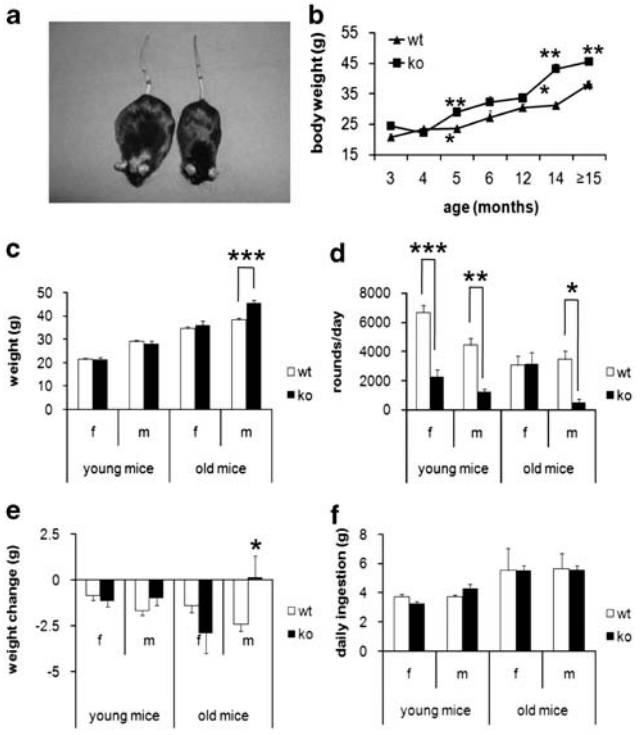

Figure 1 (**a**) Lean wild-type (WT) mouse (right) and obese serotonin transporter knockout (5-HTT ko) mouse (left). Age: 14 months, both male. (**b**) Weight development in 5-HTT ko and WT mice over time. 5-HTT ko mice have higher body weights starting at 5 months (*$P<0.05$; **$P<0.01$). (**c**) Body weight in female (f) and male (m) young and old 5-HTT ko and WT mice (mean ± s.e.m.). Old male 5-HTT ko mice had significantly higher body weight than old male WT mice (***$P<0.001$). (**d**) Treadmill rotations per day in female and male young and old 5-HTT ko and WT mice. Young 5-HTT ko female and male mice were physically less active than WT female and male mice (female: ***$P<0.001$; male: **$P=0.001$). Old 5-HTT ko male mice displayed lower motor activity than old male WT mice (**$P=0.002$). (**e**) Weight change after 6 days in the treadmill: all WT and young 5 HTT ko mice lost weight, whereas old male 5-HTT ko mice gained weight compared with old WT male mice (*$P=0.02$). (**f**) Food ingestion per day did not differ between genotypes and genders in young and old mice.

After fasting, BDNF gene expression had a trend to increase in the frontal cortex of young WT mice ($P=0.057$), but decreased in ko mice ($P=0.01$; Figure 2a). In the thalamus and in the hypothalamus, sated BDNF values were not different between genotypes in young mice. Fasting resulted only in a modest trend toward upregulation in both genotypes in the thalamus (not significant; Figure 2c) and in the hypothalamus, which reached significance in the 5-HTT ko mice ($P=0.03$; Figure 2e).

In old 5-HTT ko mice, cortical BDNF gene expression in the sated state was higher in WT compared with 5-HTT ko mice ($P=0.006$; Figure 2b). Fasting resulted in an upregulation of BDNF gene expression in both genotypes in cortex (WT: $P=0.05$; ko: $P<0.0001$; Figure 2b). In the thalamus, sated BDNF gene expression was not different between genotypes and fasting induced only a trend toward upregulation of BDNF gene expression in both genotypes (not significant; Figure 2d). In the hypothalamus, sated BDNF gene expression was also not different between genotypes. Fasting led to an upregulation of BDNF gene expression in WT mice ($P=0.04$) but not in 5-HTT ko mice (Figure 2f). There were no gender-based differences for these findings (data not shown).

The LR gene expression pattern mostly paralleled the results for BDNF (Figure 3). The main finding was that in the hypothalamus LR gene expression increased under fasting conditions in WT mice, but not in 5-HTT ko mice (see Figure 3e for young mice, $P=0.03$; Figure 3f for old mice, $P=0.001$). There were no gender-based differences for these findings (data not shown).

Immunohistochemistry for BDNF and LR on frozen sections of the hypothalamus containing the ventromedial hypothalamus paralleled the mRNA findings. The BDNF and LR immunoreactivity was higher in young WT and 5-HTT ko mice after fasting (Figures 4a–h). The BDNF and LR immunoreactivity also increased with fasting in old WT mice (Figures 4i, k, n and o), but not in old 5-HTT ko mice (Figures 4l, m, p and q). No gender-based difference was found.

Obese 5-HTT ko mice have more pronounced hepatic steatosis than WT mice

The increase in liver fat is part of the adaptive response to fasting.[21] We used oil red staining to investigate whether fasting-induced changes in liver fat are altered in 5-HTT ko mice. Fasting resulted in an increase in liver fat (Figures 5a and b) in both genotypes in young (WT: $P=0.02$, ko: $P=0.02$; Figure 5c) and old mice (WT: $P=0.001$, ko: $P=0.04$; Figure 5d). Sated old 5-HTT ko mice of both genders had significantly more liver fat than sated young ko mice ($P=0.004$) and sated old WT mice ($P=0.01$).

Metabolic serum profiles in 5-HTT ko mice

Serum leptin levels were higher in old mice of both genotypes than in young mice ($P<0.001$, Figure 6a). Old male 5-HTT ko mice had higher serum adiponectin levels than young male 5-HTT ko mice ($P=0.003$; Figure 6b). Insulin and glucose levels were not different between genotypes in young and old mice (Figure 6c and d), with a trend to increased insulin levels in old male 5-HTT ko mice. Fasting had no significant effect on any of the variables measured.

Discussion

Here we show that 5-HTT ko mice develop mature-onset obesity in a gender-dependent manner, which is associated with decreased locomotor activity but not with hyperphagia. Male 5-HTT ko mice developed obesity with increasing age, concomitantly with reduced physical activity. Expression of

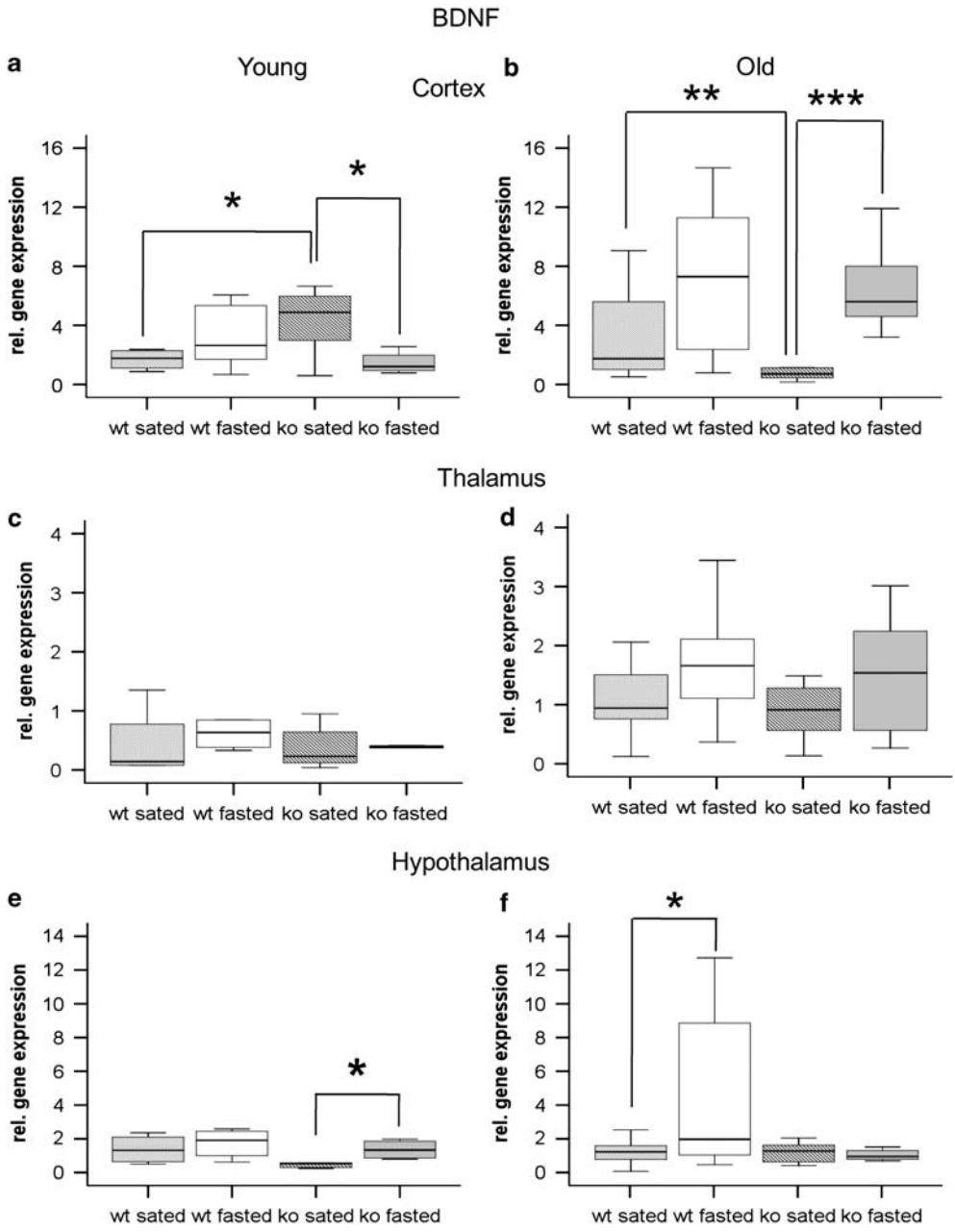

Figure 2 The boxplots illustrate the relative gene expression of brain-derived neurotrophic factor (BDNF) in the frontal cortex (**a** and **b**), thalamus (**c** and **d**) and hypothalamus (**e** and **f**) of young and old mice of both genotypes under sated and fasting conditions. The box gives the first and third quartile, the median and the minimum and maximum values measured. In young mice, fasting resulted in an upregulation of cortical BDNF gene expression in wild-type (WT) mice (n.s., not significant) and a downregulation in serotonin transporter knockout (5-HTT ko) mice (*$P = 0.01$; (**a**)). In old mice, cortical BDNF gene expression in the sated state was higher in WT mice compared with 5-HTT ko mice (**$P = 0.006$; (**b**)). Fasting caused an increase in BDNF gene expression in WT mice (n.s.) and 5-HTT ko mice (***$P < 0.0001$; (**b**)). No significant changes were found in the thalamus under fasting conditions in both age groups and genotypes (**c** and **d**). In the hypothalamus, fasting resulted in an increase of BDNF gene expression in 5-HTT ko mice in the young-age group (*$P = 0.03$; **e**), whereas in the older-age group, the fasting-induced upregulation of BDNF gene expression was only present in WT mice (*$P = 0.04$; **f**).

the weight-regulatory molecule BDNF was higher in frontal cortex of young, and lower in old 5-HTT ko mice, compared with WT mice. In contrast, the hypothalamic fasting-induced upregulation of BDNF and LR was lost in old 5-HTT ko mice.

Different animal models for late-onset obesity have been described. Obesity in heterozygous BDNF-deficient mice ($BDNF^{+/-}$) with reduced central BDNF levels is due to hyperphagia and mimics the typical phenotype of the human metabolic syndrome.[22,23] Mice deficient for the

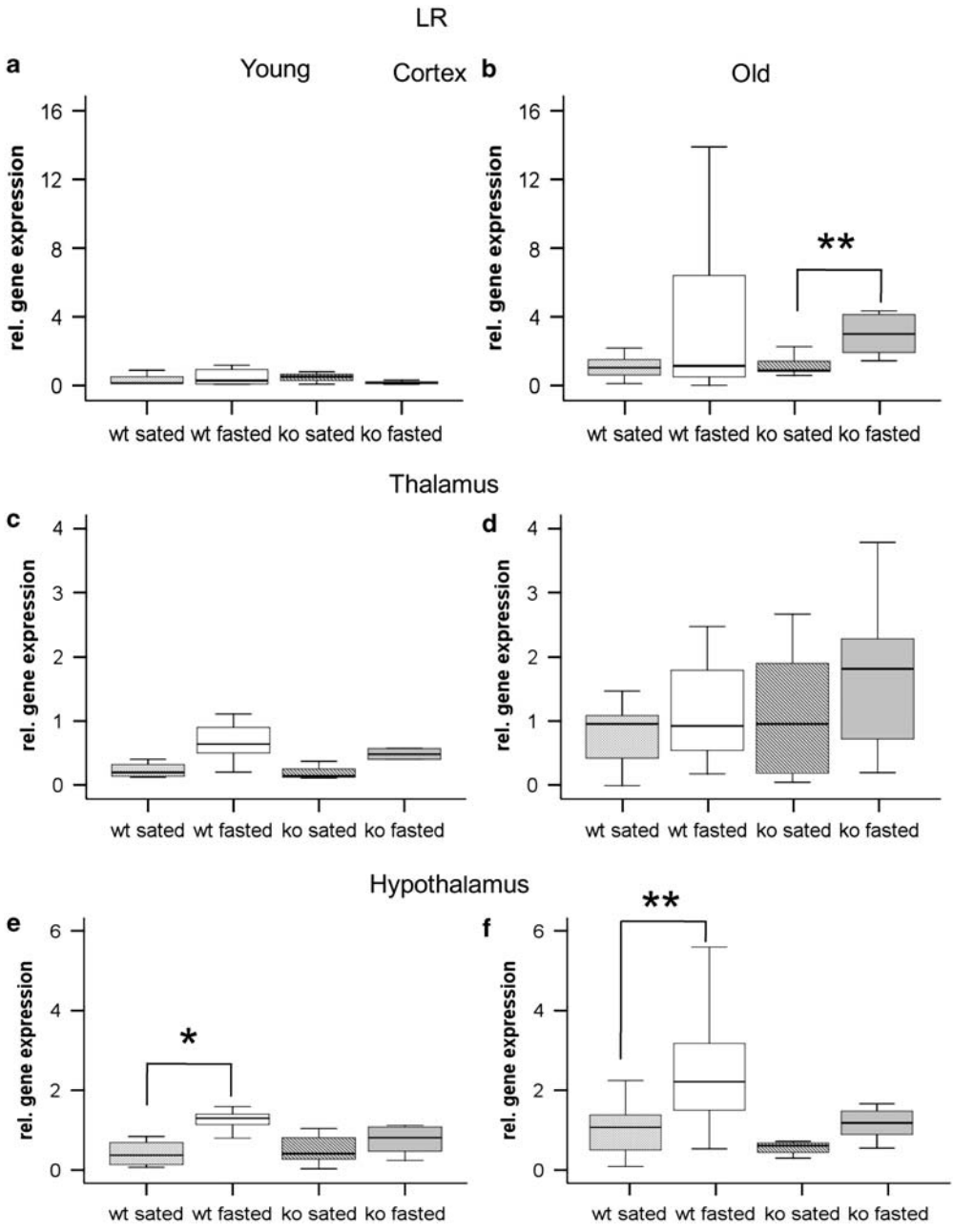

Figure 3 The boxplots illustrate the relative gene expression of leptin receptor (LR) in the frontal cortex (**a** and **b**), thalamus (**c** and **d**) and hypothalamus (**e** and **f**) of young and old mice of both genotypes under sated and fasting conditions. In the young-age group, LR gene expression in the cortex was not different between genotypes and not altered by fasting (**a**). In the older-age group, fasting caused an upregulation of LR gene expression in 5-HTT ko mice (**$P = 0.001$; (**b**)). In the thalamus, no significant differences between genotypes and no changes in LR gene expression were found after fasting in both age groups (**c** and **d**). In the hypothalamus, there was an increase of LR gene expression in young (*$P = 0.03$; **e**) and old wild-type (WT) mice (**$P = 0.001$; (**f**)).

5-HT 2C receptor become obese with age due to hyperphagia.[24,25] In contrast to these models, late-onset obesity in 5-HTT ko mice is (a) more pronounced in males and (b) associated with reduced physical activity but not with hyperphagia. According to our findings, 5-HTT ko mice become obese with age due to lack of physical activity and thus an imbalance between caloric intake and energy expenditure. In contrast to the other models for late-onset obesity, 5-HTT ko mice did not develop an overt metabolic syndrome. Serum insulin showed a trend to be increased in male 5-HTT ko mice, indicating relative insulin resistance, but glucose levels remained normal. Male 5-HTT ko mice had the capacity to upregulate adiponectin, an adipokine that is able to reverse insulin resistance.[26] This may be related to the

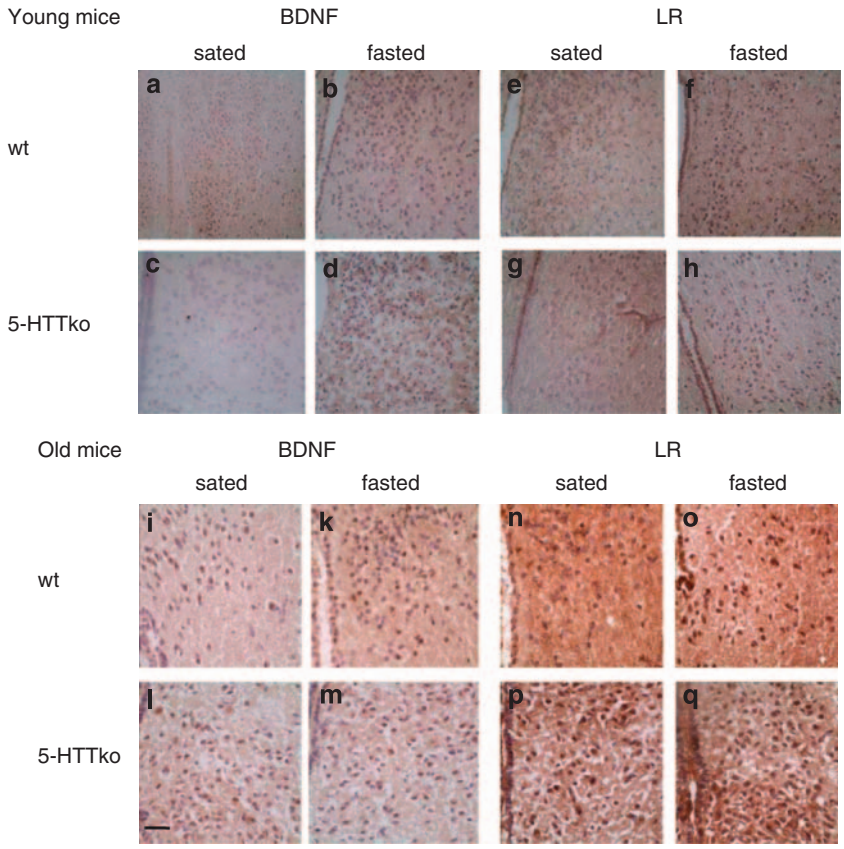

Figure 4 Immunohistochemical staining of the ventromedial hypothalamus VMH with antibodies against brain-derived neurotrophic factor (BDNF) (**a**–**d**; **i**–**m**) and leptin receptor (LR) (**e**–**h**; **n**–**q**) in young and old wild-type (WT) and serotonin receptor knockout (5-HTT ko) mice. In young WT and 5-HTT ko mice, fasting resulted in an upregulation of BDNF and LR immunoreactivity in the VMH (**b**, **d**, **f**, **h**). In old mice, this increase was not seen (**k**, **m**, **o**, **q**). Asterisks mark the third ventricle; circles around neuronal VMH cells. The bar represents 40 mm.

finding of reduced 5-HT2A receptor signaling in 5-HTT ko mice.[27,28] As antagonism to 5-HT2A receptors[29,30] increases adiponectin levels, this chain of events may protect obese 5-HTT mice from the full metabolic syndrome. Although the limitations of germline ko models compared with inducible ko strategies have to be considered, 5-HTT ko mice differ from previously described models of late-onset obesity and may serve as a paradigm for a different subset of obese humans.

Serotonin is crucial for feeding behavior[5] with prevailing anorexic effects.[9] Activation of 5-HT 1A receptors increases food intake, whereas activation of 5-HT 1B, 2A and 2C receptors attenuates feeding behavior in mice.[31–33] The 5-HTT ko mice have increased extracellular and decreased intracellular concentrations of 5-HT in the brain[11,34] and altered expression of some 5-HT receptor subtypes,[35] such that a difference in food intake compared with WT mice might have been expected. However, food intake was unaltered in 5-HTT ko mice of any age. Reasons for this difference may be that in 5-HTT ko mice the 5-HT levels are reduced, but the remaining 5-HT can act on all 5-HT receptors. Thus, reduced activation of several different receptor subtypes with opposite effects concerning feeding behavior might result in a neutral net effect.

Serotonin also influences physical activity. Depletion of cerebral 5-HT levels reduces motor activity in rats.[36] In humans, reduced central 5-HT responsivity leads to physical inactivity and promotes the development of a metabolic syndrome.[37] A decline in physical activity, in turn, is supposed to underlie middle-age weight gain.[38] Here, we observed decreased motor activity in 5-HTT ko mice already at a young age. This is consistent with the finding that the 5-HT content is reduced in the brains of these mice already at the age of 3–5 months.[10,11] However, in our cohort, 4-month-old 5-HTT ko mice were still lean in spite of reduced activity and equal food intake. An increased metabolic rate is an unlikely explanation for this finding, as body temperature was not higher than in WT mice in accordance with data from 5-HTT ko rats.[39] We therefore investigated further molecules that might be altered concomitantly with 5-HT, such as BDNF and the LR.

Brain-derived neurotrophic factor has a wide distribution in the central nervous system and is highly expressed in the ventromedial hypothalamus.[12] Its importance for feeding

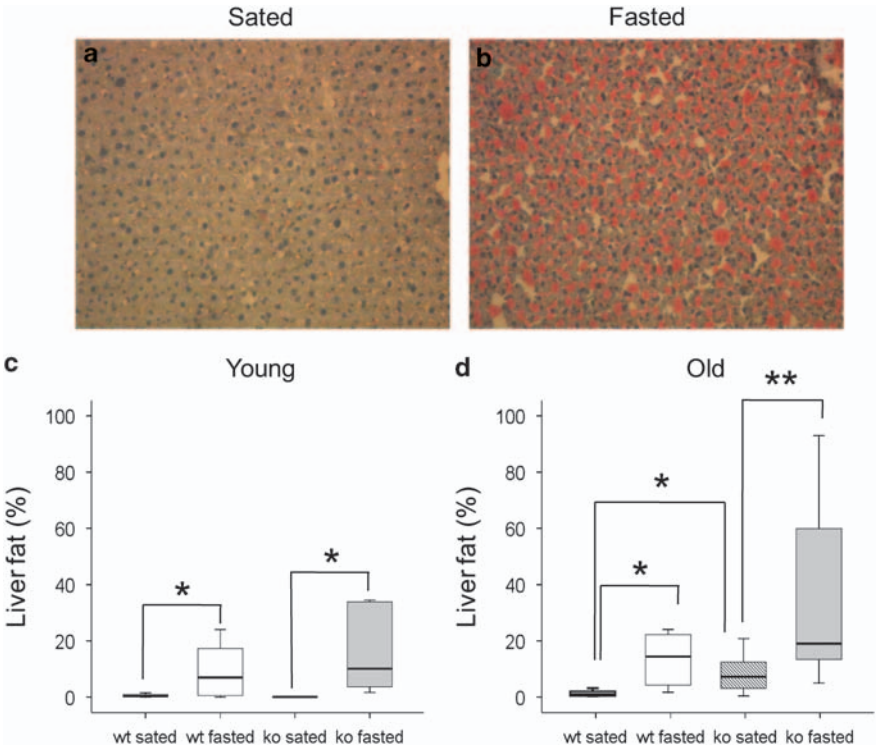

Figure 5 Representative liver sections of a wild-type (WT) mouse under sated (**a**) and fasting (**b**) conditions with oil-red staining showing steatosis after fasting. Quantitative analysis of liver fat in WT and serotonin transporter knockout (5-HTT ko) mice under sated and fasting conditions (**c** and **d**). Liver fat is demonstrated as boxplots that represent the relative fat proportion in % of the entire investigated liver section. Fasting resulted in an increase of liver fat in both genotypes in young ((**c**); $*P=0.02$) and old mice ((**d**); $*P=0.001$; $**P=0.04$). Sated old 5-HTT ko mice developed more steatosis than sated young 5-HTT ko mice ($P=0.004$) and sated WT mice ($P=0.01$).

behavior has been shown in BDNF$^{+/-}$ mice that become hyperphagic and obese with age.[22,23] Lack of the BDNF receptor TrkB also causes hyperphagia and obesity on high-fat diets.[12] Application of exogenous BDNF reduces body weight in obese diabetic (db/db) mice.[40] BDNF is upregulated in distinct brain areas during hypoglycemia, after fasting and by exercise.[20,41] In this study, young, but not old 5-HTT ko mice had a fourfold increase in BDNF mRNA level in the frontal cortex compared with WT mice. It is possible that this increase is related to their resistance to obesity at this age. In comparison to its role in feeding behavior, the role of BDNF in the control of physical activity is less well known. We speculate that through direct fiber connections between the frontal cortex and the hypothalamus,[42] cortical BDNF may act on hypothalamic TrkB receptors and thus regulate body weight. The decrease in BDNF gene expression after fasting in 5-HTT mice (in contrast to the expected increase) may indicate that the system is operating at its limits. At a higher age, the upregulation of cortical BDNF probably cannot be upheld and the compensatory mechanism against obesity may be lost, such that the mice gain weight. The BDNF levels can be increased by physical exercise, but 5-HTT ko mice do not use this mechanism, such that a long-term depletion of BDNF is likely.

Serotonergic neurons express the main BDNF receptor TrkB[13] and BDNF supports the maintenance of serotonergic neurons.[14] For example, young BDNF$^{+/-}$ mice have normal 5-HT levels, but adult BDNF$^{+/-}$ mice have reduced brain 5-HT level and loss of serotonergic axons.[23] Thus, endogenous BDNF is crucial for the maintenance of the serotonergic system. As 5-HT in turn increases the expression and secretion levels of BDNF, deficits in the regulation of BDNF would be expected in 5-HTT ko mice that have a reduced brain 5-HT content. Indeed, the expected upregulation of BDNF by fasting did not occur in the hypothalamus of old 5-HTT ko mice. One possibility to explain the observed obesity in 5-HTT ko mice might thus be the following: low 5-HT levels in 5-HTT ko mice may lead to low BDNF levels.[14] Low 5-HT levels lead to reduced locomotor activity, which in turn further reduces BDNF levels.[43] This again may decrease 5-HT activity[14,44] and result in a vicious circle promoting obesity. As outlined above, BDNF may be a protective factor against obesity in young mice.

Under normal conditions, fasting results in an upregulation of BDNF,[20] a downregulation of leptin[45] and an upregulation of LR.[19] Our results for LR regulation paralleled those for BDNF mRNA to the point that the expected hypothalamic upregulation after fasting did not occur in

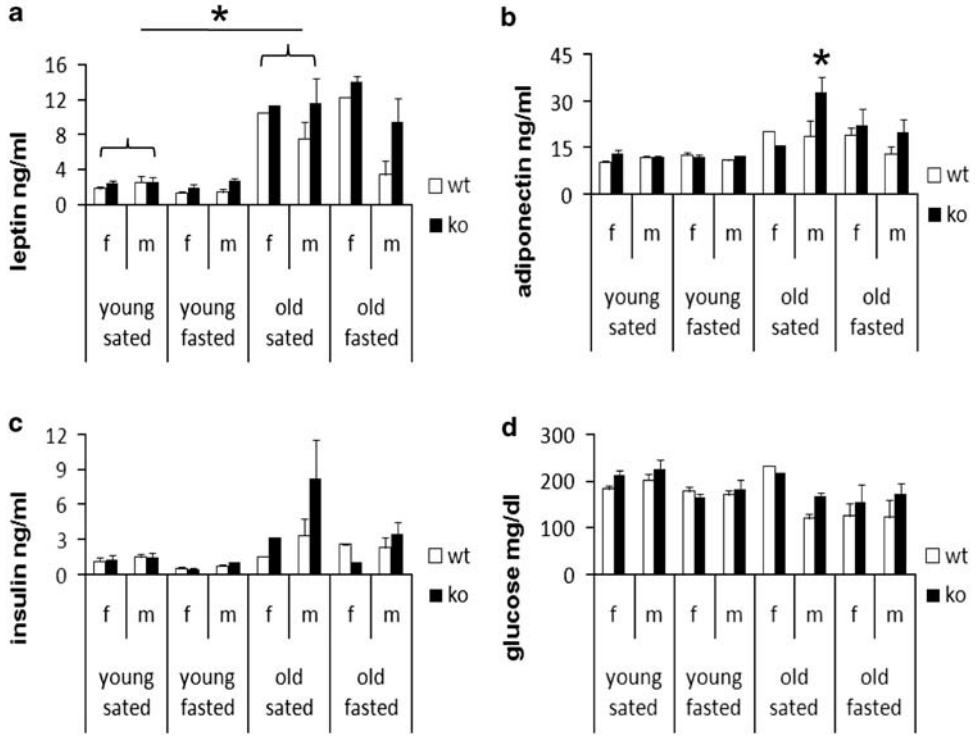

Figure 6 Serum leptin (**a**), adiponectin (**b**), insulin (**c**) and glucose (**d**) levels (mean ± s.e.m.). Leptin levels were higher in old sated serotonin transporter knockout (5-HTT ko) mice and wild-type (WT) mice compared with young mice (*$P<0.001$; (**a**)). Sated old 5-HTT ko mice had higher serum adiponectin levels than young sated 5-HTT ko mice (*$P=0.003$; **b**). Insulin (**c**) and glucose (**d**) levels were not significantly different between genotypes in young and old mice.

5-HTT ko mice. Both BDNF and leptin reduce food intake,[6] but obesity in 5-HTT ko mice was not caused by hyperphagia. Together with increased serum leptin in male 5-HTT ko mice, this may indicate relative leptin resistance.

The change in central BDNF and LR regulation found in 5-HTT ko mice was not different between males and females. However, female 5-HTT ko mice were partially protected from the obese phenotype. They developed hepatic steatosis, like the male mice, but not the age-related loss of motor activity and weight gain. This indicates the presence of further protective changes in female mice. In a previous study, female mice deficient for both the 5-HTT and BDNF were protected from the loss of 5-HT in the hypothalamus and from the reduction in TrkB receptors that occurred in male mice.[15] In these mice, also the behavioral consequences of the genotype, the increase in anxiety-like behavior, was attenuated, and estrogen was able to rescue the phenotype in male mice. Thus, estrogen may also be a protective factor against obesity in female 5-HTT ko mice.

In this study, we followed the hypothesis that obesity in old 5-HTT ko mice is due to alterations in central BDNF and LR expression. However, in the dense network of neurotransmitters and neuropeptides regulating physical activity and feeding behavior, other factors may be well of importance. It is known for instance that arcuate neurons expressing proopiomelanocortin peptides and neuropeptide Y with Agouti-related protein, are components of the hypothalamic circuits responsible for energy homeostasis.[46] These neurons also interact with hypothalamic BDNF.[47] In a recent study, serotonin was shown to alter the excitability of a subgroup of hypothalamic neuropeptide Y and proopiomelanocortin neurons in mice.[48] Ghrelin and cholecystokinin are peripherally secreted feedback factors influencing the hypothalamic regulation of metabolism. Hyperphagia can in turn regulate murine serotonin receptor expression and ghrelin secretion.[49]

The negative impact of obesity on human health is immense and one main reason is the behavioral combination of high calorie uptake and low expenditure. The major importance of physical inactivity in the pathophysiology of obesity already during adolescence is remarkable.[50] However, recent research unequivocally shows that the pathophysiology of obesity goes far beyond this simple 'high calorie-low exercise' equation. Genetic factors are increasingly recognized[51] and the key questions are (a) what induces pro-obesity behavior and (b) what protects some people with pro-obesity behavior from becoming obese? The 5-HTT ko obesity model with its link to cerebral BDNF regulation and with a protective effect of female gender may help answering these question. Associations between human 5-HT receptor polymorphisms and eating disorders or obesity have been shown.[52,53] A genetic pattern with alterations in the genes of central regulatory proteins, such as 5-HTT and BDNF, promoting physical inactivity, may increase the

susceptibility for the development of obesity also in humans.[54] Furthermore, insight into the role of protective factors as in female 5-HTT ko mice may be relevant to the prevention and treatment of obesity in humans.

Conflict of interest

The authors declare no conflict of interest.

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