Altered metabolism in the melatonin-related receptor (GPR50) knockout mouse

Elena A. Ivanova,1* David A. Bechtold,1* Sandrine M. Dupré,1 John Brennand,2 Perry Barrett,3 Simon M. Luckman,1 and Andrew S. I. Loudon1

1Faculty of Life Sciences, University of Manchester, Manchester; 2Cardiovascular and Gastrointestinal Department Discovery, AstraZeneca Pharmaceuticals, Alderley Park, Macclesfield; and 3Molecular Endocrinology Group, Division of Obesity and Metabolic Health and Aberdeen Centre for Energy Regulation and Obesity, Rowett Research Institute, Aberdeen, United Kingdom

Submitted 30 March 2007; accepted in final form 23 October 2007

Ivanova EA, Bechtold DA, Dupré SM, Brennand J, Barrett P, Luckman SM, Loudon AS. Altered metabolism in the melatonin-related receptor (GPR50) knock out mouse. Am J Physiol Endocrinol Metab 294: E176–E182, 2008. First published October 23, 2007; doi:10.1152/ajpendo.00199.2007.—The X-linked orphan receptor GPR50 shares 45% homology with the melatonin receptors, yet its ligand and physiological function remain unknown. Here we report that mice lacking functional GPR50 through insertion of a lacZ gene into the coding sequence of GPR50 exhibit an altered metabolic phenotype. GPR50 knockout mice maintained on normal chow exhibit lower body weight than age-matched wild-type littermates by 10 wk of age. Furthermore, knockout mice were partially resistant to diet-induced obesity. When placed on a high-energy diet (HED) for 5 wk, knockout mice consumed significantly more food per unit body weight yet exhibited an attenuated weight gain and reduced body fat content compared with wild-type mice. Wheel-running activity records revealed that, although GPR50 knockout mice showed no alteration of circadian period, the overall levels of activity were significantly increased over wild types in both nocturnal and diurnal phases. In line with this, basal metabolic rate (O2 consumption, CO2 production, and respiratory quotient) was found to be elevated in knockout mice. Using in situ hybridization (wild-type mice) and β-galactosidase activity (from LacZ insertion element in knockout mice), brain expression of GPR50 was found to be restricted to the ependymal layer of the third ventricle and dorsomedial nucleus of the hypothalamus. GPR50 expression was highly responsive to energy status, showing a significantly reduced expression following both fasting and 5 wk of HED. These data implicate GPR50 as an important regulator of energy metabolism.

dorsomedial hypothalamus; metabolic rate; obesity; tanycyte

The orphan melatonin related receptor (GPR50) shares ~45% homology with melatonin receptors MT1 and MT2 (20). However, the receptor does not bind melatonin (13, 20) despite the presence of a conserved histidine residue in transmembrane domain 5 of the receptor, which is a key amino acid for binding of melatonin to MT1 (9). Although the physiological function of GPR50 remains unknown, in situ hybridization studies on mouse, rat, and hamster brain sections have demonstrated an expression of the receptor in several areas associated with energy metabolism, namely the dorsomedial hypothalamic nucleus (DMN), lateral hypothalamus, and arcuate nucleus (12). Although the extent and distribution of GPR50 exhibits some degree of species specificity, high levels of expression are consistently observed in the ependymal layer of the third ventricle (12, 25).

Recently, we have shown that ependymal GPR50 expression in the seasonally breeding Siberian hamster is strongly modulated by photoperiod, in which expression is greatly reduced following exposure to short photoperiods (1). Prolonged exposure to short photoperiods leads to a dramatic reduction in body weight (typically around 40%) in these animals, mainly in the form of fat loss. The coincident reduction in expression of GPR50 in hamsters on short photoperiod raises the possibility that this receptor is involved in energy sensing and (or) regulation of metabolism. The ependymal layer of the third ventricle contains a specialized population of cells called tanycytes. These cells are thought to be involved in transmitting cerebrospinal fluid (CSF)-born signals to the parenchyma of the hypothalamus. Furthermore, these cells have been linked with thyroid hormone signaling in the brain and thyrotropin-releasing hormone (TRH) regulation. TRH is well known to be involved in thermogenesis and energy metabolism (16).

Here we demonstrate that GPR50 expression in the brain is highly responsive to energy status and describe several phenotypic characteristics of a GPR50 knockout (KO) mouse that indicate an important role for the receptor in regulating energy balance and metabolism.

MATERIALS AND METHODS

Animals. GPR50 KO mice were obtained via collaboration with AstraZeneca (Alderley Park, Cheshire, UK) who obtained the mice from DeltaGen. Mice were partially introgressed onto a C57/B6 background and maintained as a colony at the University of Manchester. The generation of these mice has been described elsewhere (1). All research using animals was licensed under the Animals Act of 1986 (Scientific Procedures) and received ethical approval from the University of Manchester animal welfare committee. Adult male mice (10–15 wk of age) were used for all experiments, housed at an ambient temperature of 20–22°C, and maintained in a 12:12-h light-dark lighting schedule unless stated otherwise. To obtain age-matched animals of controlled genetic background, heterozygote females were crossed with GPR50 KO males to obtain wild-type (WT) and KO males from within the same litter.

In situ hybridization. In situ hybridization in WT was undertaken using a murine GPR50-specific riboprobe. A fragment of 340 bases of GPR50 coding sequence was amplified using forward 5′-AAGTC-
CGAGAGCAGCCCTGTAGTATGG-3' and reverse 5'-GAAGGCGCCCTGTA-3' primers (nucleotides through 1083 to 1422) from the mouse GPR50 cDNA sequence (GenBank accession no. NM_010340) and cloned into pGEMT Easy vector (Promega, Madison, WI). The plasmid was linearized to produce antisense or sense transcripts with SP6 or T7 polymerase as appropriate. For radioactive in situ hybridization histochemistry, antisense and sense riboprobes were synthesized in the presence of [33P]UTP (MP Biomedical) and hybridization was visualized by film autoradiography (Kodak BioMax MR films; Kodak). Quantification of hybridization signal was carried out by densitometric analysis of autoradiographic films for a minimum of three sections per animal.

**X-gal staining and immunohistochemistry.** For studies of putative GPR50 protein expression, male KO mice were anesthetized with pentobarbitone sodium (0.5 g/kg ip) and perfused with cold 0.9% saline followed by cold paraformaldehyde [2% in 0.1 M phosphate buffer (PB)]. Tissues were postfixed in paraformaldehyde (2%) for 5 h at 4°C, equilibrated to 30% sucrose (in 0.1 M PB), and frozen at −80°C. Brain sections (30 μm) were collected using a freezing microtome. For X-gal staining, sections were incubated at 37°C overnight in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, 0.2% Triton X-100, and 1 mg/ml X-gal in 0.1 M PBS. X-gal staining was not observed in tissue sections collected from WT mice. Sections were then washed and processed for immunohistochemistry. Sections were blocked with 5% normal goat serum (in 0.1 M PBS, 0.2% Triton, 0.1% fatty acid-free BSA) followed by overnight incubation with an antibody raised against glucose transporter 1 (GLUT1; diluted 1:5,000; Alpha Diagnostics, San Antonio, TX), GFAP (1:1,000), or nestin (1:5,000; Abcam, Cambridge, UK). Tissue was then incubated with biotin-conjugated goat anti-rabbit IgG secondary antibody (1:400; Vector Laboratories, Peterborough, UK), followed by avidin-biotin complex (1:500; Vector Laboratories), and peroxidase activity was visualized with diaminobenzidine (Vector Laboratories). Control sections were incubated with secondary antibodies alone, which resulted in no deposition of reaction product. X-gal staining and immunohistochemical studies were carried out on multiple brain sections collected from a minimum of three mice.

**Body weight regulation, food restriction study, and determination of body fat.** Leading up to and during feeding studies, GPR50 KO and WT littermates (n = 18/group) were individually housed and main-
tained on either standard rodent chow [normal chow (NC), 3.7 kcal/g, 20% protein, 10% fat by energy; Beekay International, Hull, UK] or high-energy diet (HED, 4.5 kcal/g, 20% protein, 45% fat by energy; Harlan, Indianapolis, IN). Body weight regulation, food restriction study, and determination of body fat content in KO and WT mice (of body fat. multiple brain sections collected from a minimum of three mice. Laboratories). Control sections were incubated with secondary anti-
peroxidase activity was visualized with diaminobenzidine (Vector Laboratories). For X-gal staining, sections were incubated at 37°C overnight in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, 0.2% Triton X-100, and 1 mg/ml X-gal in 0.1 M PBS. X-gal staining was not observed in tissue sections collected from WT mice. Sections were then washed and processed for immunohistochemistry. Sections were blocked with 5% normal goat serum (in 0.1 M PBS, 0.2% Triton, 0.1% fatty acid-free BSA) followed by overnight incubation with an antibody raised against glucose transporter 1 (GLUT1; diluted 1:5,000; Alpha Diagnostics, San Antonio, TX), GFAP (1:1,000), or nestin (1:5,000; Abcam, Cambridge, UK). Tissue was then incubated with biotin-conjugated goat anti-rabbit IgG secondary antibody (1:400; Vector Laboratories, Peterborough, UK), followed by avidin-biotin complex (1:500; Vector Laboratories), and peroxidase activity was visualized with diaminobenzidine (Vector Laboratories). Control sections were incubated with secondary antibodies alone, which resulted in no deposition of reaction product. X-gal staining and immunohistochemical studies were carried out on multiple brain sections collected from a minimum of three mice.

**RESULTS**

**Reduced weight gain in mice lacking GPR50.** GPR50 KO mice exhibited significantly lower body weights in the weeks following weaning and by 10 wk of age were significantly lighter (n = 19–20/genotype; WT: 29.3 ± 0.4 g; KO: 27.4 ± 0.3 g; P < 0.01; Fig. 1A). Weight gain over the following 5 wk (10–15 wk of age) was similar in both genotypes in mice maintained on normal chow (NC). To examine susceptibility to diet-induced obesity, WT and KO mice were placed on a HED for 5 wk (from 10 wk of age, n = 10–11/genotype on NC and 17–18/genotype on HED). At the end of the 5 wk, WT mice eating HED were significantly heavier than both WT mice maintained on NC and GPR50 KO mice placed on HED (Fig. 1, A and B). In contrast, change of diet did not lead to a significant difference in body weights between GPR50 KO mice maintained on NC or HED (WT-NC: 32.5 ± 0.7 g; WT-HED: 38.5 ± 1.2 g; P < 0.05; KO-NC: 30.6 ± 0.7 g; KO-HED: 33.7 ± 0.9 g; P < 0.05; 2-way ANOVA; Fig. 1A). Interestingly, the attenuated weight gain in GPR50 KO mice (Fig. 1C) occurred despite the fact that these mice consumed significantly more food per unit body weight over the 5-wk period (Fig. 1D). Therefore, differences in body weight gain in mice lacking GPR50 are not because of food intake, suggesting that these mice exhibit an increased resistance to diet-induced obesity and (or) decreased energy efficiency (body weight gained divided by food consumed; Fig. 1E).

Analysis of body fat carried out on the mice following 5 wk of NC or HED revealed a reduction in body fat content in the KO mice (n = 4–5/group). Specifically, GPR50 KO animals fed HED for 5 wk were found to have 31.9 ± 2.1% body fat compared with 39.1 ± 1.4% in WT. This percent body fat equals a mean fat weight of 10.5 g in KO mice vs. 15.6 g in the WT mice (based on wet tissue weight) and accounts for 70.1%
of the weight difference observed in the genotype weight difference following HED (mean fat weight: WT-NC: 8.1 ± 0.6 g fat; KO-NC: 5.2 ± 0.2 g; WT-HED: 15.7 ± 0.5 g; KO-HED: 10.5 ± 0.8 g; 2-way ANOVA, \( P < 0.05 \) between WT and KO on HED). Therefore, the attenuated weight gain in the KO mice appears to be due primarily to a reduction in body fat and supports the suggestion that GPR50 KO mice are partially resistant to diet-induced obesity. Plasma leptin levels were also reduced in the GPR50 KO mice (Table 1), although these differences did not reach statistical significance on either diet. As expected, plasma leptin levels were significantly correlated with body fat content in all mice (\( r^2 = 0.80; \ P < 0.001 \), spearman correlation analysis). Plasma glucose and corticosterone levels were examined in KO and WT mice fed NC or HED, as well as in mice that had been fasted for 24 h (Table 1). No significant differences were observed between the two genotypes, although plasma corticosterone levels were consistently higher raised in the KO mice.

Interestingly, GPR50 KO mice lost significantly less body weight during a 36-h fast when compared with WT mice (KO: 14.0 ± 0.7% body weight loss; WT: 19.4 ± 0.7%; \( P < 0.01 \) Student’s \( t \)-test), suggesting an altered response to negative energy balance in the KO mice.

**Altered activity and metabolic rate in GPR50 KO mice.** Measurements of wheel-running activity patterns revealed that there were no significant differences in circadian periods between the two genotypes (WT: 23.8 ± 0.17 h; KO: 23.6 ± 0.13 h). However, the overall intensity of activity, as measured by total number of wheel revolutions, was significantly greater for KO mice during both the light phase (typically a mouse’s inactive period; WT: 397 ± 67 revolutions; KO: 633 ± 108, \( P < 0.05 \)) and over the entire 24-h period (WT: 9,979 ± 356; KO: 13,487 ± 1,123, \( P < 0.05 \); Fig. 2, A and B).

Measurements by indirect calorimetry of metabolic rate were made over a 4-day test period. Analysis of the data accumulated during the light or dark periods revealed that GPR50-deficient mice showed elevated basal metabolic rate as assessed by \( V_{O2} \) (Fig. 3A) and \( V_{CO2} \) (Fig. 3B), both of which were elevated in GPR50 KO mice during both the light and dark phases of the day. These differences reached statistical significance during the light phase (\( V_{O2} \): WT: 2,967.3 ± 66.4 ml·kg \(^{-1} \)·h \(^{-1} \); KO: 3,215.0 ± 70.6, \( P < 0.05 \); \( V_{CO2} \): WT: 2,766.4 ± 59.9 ml·kg \(^{-1} \)·h \(^{-1} \); KO: 3,073.1 ± 68.7, \( P < 0.05 \)). Although not significantly different between the genotypes, RQ was elevated in GPR50 KO mice during both the light and dark phases of the day, likely reflecting the increased food consumption by these mice compared with WT (Fig. 3C). GPR50 expression is highly localized and responsive to energy status. In WT mice, in situ hybridization histology revealed strong GPR50 mRNA expression in the ependymal layer of the third ventricle and DMN (Fig. 4A). Ependymal expression was confined to the ventricular wall caudal in the retrochiasmatic region. Probing of GPR50 KO brain sections with antisense riboprobe or WT brain sections with sense riboprobe produced no detectable hybridization signal (data not shown). GPR50 mRNA expression was also assessed under conditions of altered energy status (Fig. 4). Specifically, food...
restriction (36 h) caused a significant decrease in GPR50 expression in both the ependymal cells and DMN (Fig. 4B), which recovered to constitutive levels following 12 h of refeeding (Fig. 4C). Interestingly, mice maintained on HED for 5 wk exhibited a similar downregulation of GPR50 expression (Fig. 4D), suggesting that GPR50 expression is responsive to both negative and positive energy balance.

Putative expression of GPR50 protein was visualized in the ependymal cells of male GPR50 KO mice by staining for β-galactosidase activity (Fig. 5, A–C). X-gal reaction product was observed in a highly restricted population of cells lining the third ventricle. In contrast to the in situ hybridization results, only scattered X-gal positive cells were observed in the DMN. This pattern of X-gal staining was also observed in female heterozygote mice. In an attempt to characterize GPR50-expressing cells in the ependymal, X-gal staining was combined with immunohistochemistry for cell-specific markers. X-gal-containing cells did not appear to coexpress the glial astrocyte marker GFAP (Fig. 5D), GLUT1 (a marker for α- and β-tanyctes; Fig. 5, E and F), or the progenitor cell associated intermediate filament nestin (Fig. 5, G and H). It must be acknowledged that the use of β-galactosidase activity is not a definite marker for GPR50 protein expression. This method has a relatively low sensitivity, and the fact that the cells do not express functional GPR50 may alter other characteristics of the cells.

**DISCUSSION**

Our results strongly implicate the orphan receptor GPR50 in the regulation of energy metabolism. Mice lacking GPR50 exhibited reduced body weight and showed a partial resistance to diet-induced weight gain when fed a hypercaloric, high-fat diet. During 5 wk of HED, GPR50 KO mice gained significantly less weight than WT mice, which was due predominantly to reduced fat content in the KO mice (31.9 vs. 39.1% body fat). Importantly, weight gain was attenuated in KO mice despite the fact that they consumed more food per gram of body mass. GPR50 KO mice were found to be hyperactive when placed in wheel-running cages, a characteristic that may...
contribute to their reduced weight gain and partial resistance to diet-induced obesity. We have not explored whether other aspects of behavior are altered in these mice, but it was also noticeable that KO mice were generally more active and harder to handle than WT counterparts. Furthermore, elevated levels of circulating corticosterone were consistently observed in the GPR50 KO mice, raising the possibility that these animals have a heightened stress response. In line with the running-wheel activity, VO2 and VCO2 levels were found to be elevated in GPR50 KO mice. Because the mice were not simultaneously monitored for activity during the calorimetric studies, we do not know whether the increased metabolic rate in KO mice arises as a direct result of altered activity patterns or is reflective of more fundamental alterations in basal metabolic rate.

The localization of GPR50 within the brain using β-galactosidase activity and in situ hybridization revealed a highly restricted pattern of expression, confined to a subset of the ependymal cells of the third ventricle (A). Fasting mice for 36 h caused a profound decrease in GPR50 expression (B), which recovered following 12 h of refeeding (C). Reduced GPR50 expression was also reduced following 5 wk of HED (D). Quantification of the hybridization signal (E, n = 6/group) showed a significant reduction in signal in the ependymal layer of fasted animals when compared with either normally fed controls (*P < 0.05) or reed mice (#P < 0.05, 1-way ANOVA). Mice maintained on HED for 5 wk also exhibited a significant decrease in GPR50 expression in the ependyma (+P < 0.05). Levels of GPR50 mRNA were reduced similarly in the DMN.

Previously shown that ependymal GPR50 expression exhibits seasonal variation in the Siberian hamster (1) and may be implicated in seasonal changes in energy sensing and metabolism. Specifically, GPR50 expression is strongly reduced following prolonged (10 wk) exposure to short day lengths, a condition that leads to a dramatic reduction in adiposity in this species.

The DMN is known to be involved in thermoregulation, energy balance, and body weight regulation. Interestingly, bilateral lesioning of the DMN in rats leads to body weight loss but maintenance of normal body composition (2). Furthermore, DMN-lesioned rats placed on a high-fat diet gain more weight than those maintained on NC but do not become as obese as sham-operated rats (4). Diet-induced obese rats exhibit an increased expression of c-fos (taken as a measure of increased neuronal activity) in the DMN compared with obesity-resistant rats (26). The similarities between DMN-lesioned rats and our KO mice suggest that the loss of GPR50 signaling in the DMN may impair energy homeostasis mechanisms. The principle hypothalamic target for efferent projections from the DMN is the paraventricular nucleus, and some of these projections are known to interact with corticotropin-releasing hormone (CRH) and TRH expressing neurons (11, 18, 24). Both CRH and TRH have well-characterized effects on food intake and body weight regulation. Although GPR50 is strongly expressed in the DMN with an in situ probe, the lack of X-gal staining in the DMN of KO mice may be because of reduced sensitivity of the method (vs. in situ hybridization), disruption of some DMN-specific
regulatory sequence in the GPR50 gene in the KO, or an inability of DMN neurons to properly translate the LacZ transcripts. It is also possible that GPR50 protein is not expressed in the DMN because of some posttranscriptional events.

The other major population of GPR50-expressing cells in the brain lies within the ependymal layer of the third ventricle. This region of the ventricle is also populated by a specialized group of glial cells called tanycytes. Tanycytes can be classified into four recognized subtypes (α1, α2, β1, and β2) based principally on their spatial orientation within the ependymal layer and have been implicated in relaying signals born within the CSF to hypothalamic neurons (15, 21). The overall expression pattern of GPR50 suggests that these cells may be tanycytes. However, GLUT1, which is considered as a marker for α1 and α2 tanycytes, and to a lesser extent β1 tanycytes (3, 19), was not found to be colocalized with GPR50, and the precise identity of the GPR50-expressing ependymal cells remains to be determined. It is possible that GPR50-dependent signaling is involved in CSF-neuron communication, possibly responding to metabolites within the CSF such as glucose or fatty acid. Ependymal tanycytes are also key cells involved in the deiodination of thyroxine to the active metabolite triiodothyronine via type II deiodinase (D2; see Refs. 6 and 14). Similarly to GPR50, D2 gene expression is sensitive to nutritional status (10). We have previously shown that, like GPR50, the expression of the intermediate filament nestin in the third ventricle ependyma is seasonally regulated in the Siberian hamster (1). However, the expression pattern of GPR50 (as shown by X gal staining) and nestin in the mouse third ventricle suggests that these two proteins are not coexpressed in the same cells.

Drew and coworkers (12) reported a more extensive range of GRP50 expression in the brain using radiolabeled riboprobes; however, many neural structures were only apparent after long film exposure times. In our study, we have found a more restricted expression pattern of GPR50 in the mouse brain. GPR50 expression has also been demonstrated in several peripheral body organs, including, eye, skin, ovaries, testis, and intestine. The expression of GPR50 in peripheral tissues, including within the gastrointestinal tract, raises the possibility that KO mice have altered digestive function, which could contribute to the metabolic phenotype of these mice. GPR50 is distantly related to the melatonin receptors, and it is possible that melatonin itself may play a role. A recent study using cell lines has proposed that GPR50 may heterodimerize with melatonin receptors in response to melatonin (17). There are, however, several reasons why such a signal transduction path-

Fig. 5. Ependymal expression of GPR50. With the use of β-galactosidase activity in knockout mice, putative GPR50 protein expression was detected in the ependymal layer of the third ventricle (in A–C rostral to caudal sequence). However, only scattered X-gal staining was observed in cells of the DMN (C, arrow). X-gal staining (blue staining) did not appear to be localized to ependymal cells expressing GFAP (D, brown staining), GLUT1 (E and F, brown staining), or nestin (G and H, brown staining).
way is unlikely in this case. Melatonin is not synthesized in the mouse used in these experiments (C57/B6), as with the majority of mouse strains, and melatonin receptors have not been identified in the ependymal cell layer (22, 23).

A recent study by Bhattacharyya et al. (5) of an obese cohort of humans identified sequence variations in GPR50, including an insertion of four amino acid residues (TTGH) at position 501 (COOH-terminal tail) and several single-nucleotide polymorphisms that show significant associations with elevated circulating triglyceride and high-density lipoprotein levels, suggesting a role for this receptor in the regulation of lipid metabolism. Taken together with our current data on the GPR50 KO mouse, these findings suggest a novel role for this orphan receptor in energy sensing and metabolic regulation.

ACKNOWLEDGMENTS

We thank David Brown (Rowett) for the lipid analysis on whole animal carcasses.

GRANTS

This work was supported by grants from the Biotechnology and Biological Sciences Research Council (A. S. I. Loudon, S. M. Luckman) and Scottish Executive Environment and Rural Affairs Department (P. Barrett).

REFERENCES