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Effects of cell-type specific leptin receptor mutation on leptin transport across the BBB

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Abstract

The functions of leptin receptors (LRs) are cell-type specific. At the blood-brain barrier, LRs mediate leptin transport that is essential for its CNS actions, and both endothelial and astrocytic LRs may be involved. To test this, we generated endothelial specific LR knockout (ELKO) and astrocyte specific LR knockout (ALKO) mice. ELKO mice were derived from a cross of Tie2-cre recombinase mice with LR-floxed mice, whereas ALKO mice were generated by a cross of GFAP-cre with LR-floxed mice, yielding mutant transmembrane LRs without signaling functions in endothelial cells and astrocytes, respectively. The ELKO mutation did not affect leptin half-life in blood or apparent influx rate to the brain and spinal cord, though there was an increase of brain parenchymal uptake of leptin after in-situ brain perfusion. Similarly, the ALKO mutation did not affect blood-brain barrier permeation of leptin or its degradation in blood and brain. The results support our observation from cellular studies that membrane-bound truncated LRs are fully efficient in transporting leptin, and that basal levels of astrocytic LRs do not affect leptin transport across the endothelial monolayer. Nonetheless, the absence of leptin signaling at the BBB appears to enhance the availability of leptin to CNS parenchyma. The ELKO and ALKO mice provide new models to determine the dynamic regulation of leptin transport in metabolic and inflammatory disorders where cellular distribution of LRs is shifted.

Keywords

Leptin; BBB; Transport; Endothelial cells; Astrocytes; CNS effects

Introduction

It was first shown in 1996 that leptin is transported across the blood-brain barrier (BBB) by a saturable transport system [5]. This concept is physiologically important, because the 16 kD polypeptide leptin is mainly produced by adipocytes in the periphery. The BBB and the

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permeability barrier between circumventricular organs and the main brain parenchyma prevent diffusion of large hydrophilic molecules [4,9,22,23,38]; direct permeation of leptin to most CNS targets is regulated by the saturable transport system. At the BBB level, leptin transport is mainly mediated by its specific receptors that show abundant expression in cerebral microvessels [8,10,15,30]. In cerebral endothelial cells that are the major component of the BBB, receptor subtype ObRa (LRa) is most abundant and generally considered a transporting receptor, whereas ObRb (LRb) and ObRe (LRe) may also play a role. Among the receptor subtypes, ObRb activates Janus kinase 2 and Signal Transducer and Activation of Transcription (STAT)-3, whereas all membrane bound receptors can activate STAT1, phosphoinositol-3 kinase, mitogen activated protein kinases, cyclin dependent kinase-5, and others. Understanding of the leptin system at the BBB is based on studies from several groups involving cellular assays with full-length [16,35–37] or mutant receptors [7,34], and analyses of obese mice with hyperleptinemia or hyperlipidemia [2,3]. However, receptor deficiency can be compensated without loss of leptin transport, as seen in obese db/db mice lacking the ObRb receptor [25], in obese Koletsky rats [6,24], and in changes in levels of ObR expression during development [30] and the progression of obesity [29].

Adding to the complex picture, astrocytes also express ObR mRNA and protein [19] which show robust regulatory changes in obesity and neuroinflammation. Astrocytes are an integral part of the BBB. Astrocyte end feet not only reinforce the structural integrity of the BBB by anatomical interactions with microvessels, but astrocytes also regulate gliovascular coupling [1,20]. We have shown in a Transwell cellular model that the levels and subtypes of astrocytic leptin receptors affect leptin permeation across the endothelial monolayer. We have also shown that leptin transport may not be receptor subtype-specific, given sufficient level of expression of the membrane bound receptors [36], though the soluble receptor ObRe inhibits leptin transport both in-vitro and in-vivo [35]. Furthermore, truncated leptin receptors with a short cytoplasmic tail or no intracellular sequence remain effective in mediating leptin endocytosis [34]. This indicates dissociation between cellular trafficking and signaling. However, it has not been shown in-vivo whether endothelial specific or astrocyte specific ObR mutation play differential roles in leptin transport across the BBB. This issue is particularly pertinent in neonatal development [30] and pathological conditions [17,29] where cell- type specific ObR regulation occurs. To address the respective roles of endothelial and astrocytic ObRs in leptin transport across the BBB, we generated endothelial specific leptin receptor mutant (ELKO) and astrocyte specific leptin receptor mutant (ALKO) mice, and analyzed their effect on leptin transport.

Materials and Methods

Generation of ELKO mice

LR-floxed mice were generated in the Chua lab [26,27]. The loxP sites are located in Intron 16 and Exon 17' that encode the 3' terminus of LRC. The two loxP sites flank exon 17 that encodes the BOX 1 domain required for JAK-STAT signaling. Deletion of the flanked sequence disrupts the terminal exon of each membrane-bound LR isoform by a frameshift of subsequent coding sequences, and generates a short sequence of 14 amino acids (aa) immediately following Exon 16 without known signaling functions (LKHLSIFLPSMQNQ) [26]. The LR-floxed mice were backcrossed with C57 for more than 6 generations in the Obici lab before arrival at our BBB Group.

The LR-floxed mice on a C57 background were crossed with endothelial specific promoter Tie2-Cre recombinase transgenic mice (Tie2^{cre/wt}) from Jackson Laboratory (Bar Harbor, ME), also on a C57 background. The resulting LR^{loxP/+}/Tie2^{cre/wt} mice were further crossed with LR^{loxP/loxP} mice to yield an F2 generation of LR^{loxP/loxP}/Tie2^{cre/wt}, or ELKO

mice. The F2 generation also contains LR^{loxP/loxP}, LR^{loxP/wt}, and LR^{loxP/+Tie2^{cre/wt}} that were used as controls. For genotyping, DNA was prepared from tail snips of all mice at the time of weaning (3 weeks old) and used as the template for PCR. The primers for the wildtype and mutant mice are listed in Table 1. Tissue-specific deletion of membrane-bound ObR was further verified by qPCR quantification of total RNA isolated from cerebral microvessels obtained by capillary depletion [28,39], cerebral cortex, and peripheral organs.

Generation of ALKO mice

To generate astrocyte-specific deletion by cre-loxP approach, we chose the glial fibrillary acidic protein (GFAP) promoter. Although a small percent of neural progenitor cells is also GFAP(+), the crossbreeding of GFAP-cre mice (Jackson Laboratory, FVB background) with LR-floxed mice (FVB background, maintained in the Chua lab for many years) assures deletion of membrane-bound ObR in adult astrocytes. Since the two loxP sites flanked Exon 17 that encodes the cytoplasmic domain, the resulting mutant receptor ($\Delta 17$) remains membrane-bound but has no known signaling function because of the absence of Box 1 (shared by ObRa, ObRb, ObRc), Box 2, and Box 3 (unique to ObRb). The F1 mice arising from a cross of GFAP-cre/+ heterozygote transgenics with ObR-flox/flox homozygotes were then crossed with ObR-flox/flox homozygotes to obtain the F2 generation. The primers for genotyping are listed in Table 1. The sizes of the PCR products are: 339 bp for floxed LR, 289 bp for wildtype LR, and 208 bp for $\Delta 17$. Since the two genes are on different chromosomes, there is a one-fourth chance of obtaining true ALKO mice that contain PCR products of GFAP-cre, $\Delta 17$ sequence, and floxed ObR in genotyping. Weaning and genotyping were performed when the mice were 21 days old, and randomly housed, with ALKO mice maintained in the same cages, as the control mice.

Besides qPCR verification of ObR mRNA in primary astrocytes obtained from wildtype littermates and ALKO mice, immunohistochemistry (IHC) was performed with a long isoform ObRb specific antibody targeting the C-terminus epitope (R & D Systems, Minneapolis, MN). Astrocytes were immunostained with a polyclonal antibody against GFAP. Co-localization of ObRs with astrocytes was determined by confocal microscopic analysis, as described in detail previously [17]. Negative controls confirming specificity of the staining included sections with omission of primary antibodies and preadsorption of ObR by overnight incubation with blocking peptides.

BBB transport assays in the ELKO mice

Following a protocol approved by the Institutional Animal Care and Use Committee, 3 month (m) old ELKO and wildtype littermate controls (n = 8/group) were used for BBB transport of ¹²⁵I-leptin after anesthesia, as described previously [21,29]. A vascular permeability marker ¹³¹I-albumin was included as a negative control. To determine the influx rate from blood to brain, the anesthetized mice received intravenous injection of ¹²⁵I-leptin and ¹³¹I-albumin (about 1 μ Ci each in 100 μ l of lactated Ringer's solution containing 1 % bovine serum albumin) at time 0, and were decapitated at different time points 1 – 20 min later, with one mouse representing each time point. Blood was collected from the right common carotid artery immediately before decapitation. Brain was dissected into cerebral cortex, hippocampus, hypothalamus, striatum, and the rest of the brain, based on known functions of leptin in these regions. Spinal cord was separated into cervical, thoracic, and lumbar segments. CNS tissue and serum radioactivity was determined by measurement in a γ -counter with a dual channel program, and the influx rate was determined from the linear regression relationship between the tissue/serum ratio of radioactivity and exposure time, a theoretical steady-state time if blood concentration of leptin had remained constant [21].

To further determine compartmental distribution of ^{125}I -leptin and ^{131}I -albumin in the brain, a capillary depletion study was performed on cortical samples of the above mice after in-situ brain perfusion, as described previously [32,39]. This dextran density centrifugation procedure allows separation of microvessels and parenchyma in cerebral cortex. The influx of ^{125}I -leptin and ^{131}I -albumin into cerebral cortex and the remaining tracers in the capillary fractions were compared between the ELKO and control groups.

Separate groups of male ELKO ($n = 5$) and floxed littermate controls ($n = 6$) were studied at 8 m of age. To avoid interference from possible changes in ObRe (LRe) and other blood-borne factors, in-situ brain perfusion was performed with serum-free buffer as described previously [32]. Brain tissue uptake after 5 min of perfusion at 2 ml/min was determined, with 2 min of pre-perfusion to clear the vascular space and 1 min of post-perfusion to remove any radioactively labeled tracers remaining in the vasculature. Capillary depletion was performed, and the difference as a result of different compartment (parenchyma vs capillary) and mutation (ELKO vs wildtype) was determined by two-way analysis of variance. The uptake at 5 min was expressed as (^{125}I -leptin – ^{131}I -albumin) brain/perfusate ratio after normalization.

BBB transport assays in the ALKO mice

As described above, multiple-time regression analysis was performed to determine leptin transport from blood to brain and spinal cord in groups of ALKO and littermate controls. To determine the amount of the radioactivity during the study period that remained as intact ^{125}I -leptin, acid precipitation was performed on serum and supernatant of brain homogenates. The linear regression of brain uptake over time between the ALKO and wildtype groups was analyzed by the least squares method with the Prism GraphPad program.

Results

1. Confirmation of endothelial specific LR mutation in ELKO mice

Crossbreeding of the F1 generation of $\text{LR}^{\text{loxP}/+}/\text{Tie2}^{\text{cre}/\text{wt}}$ mice with $\text{LR}^{\text{loxP}/\text{loxP}}$ mice produced 4 genotypic patterns determined from tail samples: (1) ELKO mice ($\text{LR}^{\text{loxP}/\text{loxP}}/\text{Tie2}^{\text{cre}/\text{wt}}$) that contain polymerase chain reaction (PCR) products for the transgene of Tie-2 cre recombinase (100 bp), the floxed LR (339 bp), and the mutant LR (without the functional Exon 17 sequence encoding Box 1 of cytoplasmic LR, see Materials and Methods, abbreviated as $\Delta 17$ sequence); (2) $\text{LR}^{\text{loxP}/\text{loxP}}$ (floxed) mice that contain PCR products only for Tie2 wildtype ($\text{Tie2}^{\text{wt}/\text{wt}}$, 324 bp) and floxed LR; (3) $\text{LR}^{\text{loxP}/+}/\text{Tie2}^{\text{cre}/\text{wt}}$ (heterozygotes, Fig. 1, lane 4) that contain PCR products for Tie-2 cre, floxed LR, and wildtype LR; and (4) $\text{LR}^{\text{loxP}/+}/\text{Tie2}^{\text{wt}/\text{wt}}$ mice (heterozygotes) that contain PCR products for Tie2 wildtype, floxed LR, and wildtype LR (289 bp) (Fig. 1). The endothelial specific deletion of full-length LR was confirmed by quantitative RT-PCR in purified microvessels and control tissues by use of primers targeting the sequence encoded by Exon 17 (shared by membrane-bound LR isoforms) and the sequence specific for LRb. Since the floxed mice and heterozygotes did not show differences in body weight and fat composition in preliminary studies, the floxed mice were used as the main littermate controls for functional assays.

2. Influx rate and parenchymal uptake of ^{125}I -leptin in ELKO and wildtype mice

The ELKO mutation did not change the serum half-life of ^{125}I -leptin in comparison with the wildtype control mice. Multiple-time regression analysis showed that the influx rate of ^{125}I -leptin from blood to brain was not significantly different between the ELKO and floxed mice studied when 3 m old ($n = 8/\text{group}$) (Fig. 2A). When dissected into different regions,

there was no difference of the apparent influx rate in either brain or spinal cord between the two groups of mice (Fig. 2B). The cerebral cortex was dissected from whole brain to avoid circumventricular regions, and further separated into parenchymal and capillary compartments by dextran-based density centrifugation (capillary depletion method). Capillary depletion analysis of the ELKO mice showed a non-significant increase of influx rate but lower initial volume of distribution than with the wildtype controls (Fig. 2C). In both groups of mice, parenchymal uptake was significantly higher than capillary retention at each time point.

3. Increased parenchymal uptake of leptin shown by in-situ brain perfusion

Two groups of ELKO and floxed littermate control mice were studied at 8 m of age ($n = 5-6$ /group) to determine whether the LR mutation induced a difference in parenchymal uptake and residual capillary retention after 5 min of in-situ brain perfusion with ^{125}I -leptin and ^{131}I -albumin. With pre- and post-perfusion procedures with buffer only, most of the loosely adherent radioactive tracers would have been removed; nonetheless, we performed a normalization step by subtraction of the albumin space to assure the accuracy of assessment of leptin uptake. Unexpectedly, the ELKO group had a significantly higher uptake of leptin than the wildtype mice in both brain parenchyma and cerebral microvessels. Altogether, the results indicate that more leptin reached the brain of ELKO mice after 5 min of perfusion than in the wildtype controls (Fig. 3).

4. Genetics and protein expression studies of the ALKO mice

ALKO mice of either gender were visually indistinguishable from littermate controls in both the neonatal and adult periods of life. There were no changes in gross motor function, muscular and skeletal development, adiposity, or feeding behavior. The weight of the brain and spinal cord was not significantly different from littermate controls ($n = 8$ /group), and there was no cell loss or architectural changes by histological examination. Successful deletion of wildtype LR with emergence of the mutant $\Delta 17$ ObR was shown by PCR genotyping not only in tail DNA (Fig. 4A), but also in cultured primary astrocytes from the ALKO mice (Fig. 4B).

In the brain of the ALKO mice, successful deletion of ObRb from astrocytes was shown by IHC with an ObRb specific antibody targeting the C-terminus of the receptor (R & D Systems, Minneapolis, MN). This was most apparent in tanocytes along the walls of the third ventricle, whereas neuronal ObRb was unaffected (Fig. 5).

5. BBB characteristics of the ALKO mice

Subtypes and levels of LR in astrocytes affect the permeation of leptin across cerebral endothelial cell monolayer, an approximate in-vitro model of the BBB [18]. To determine whether deletion of astrocytic membrane-bound LR affects leptin transport across the BBB in-vivo, multiple-time regression analysis was performed on groups of ALKO and wildtype littermate controls ($n = 8$ /group). The ALKO mice did not show a significant difference in the influx rate or initial volume of distribution of ^{125}I -leptin in the brain in comparison with the control mice (Fig. 6A). Similarly, in different regions of the spinal cord (cervical, thoracic, and lumbar segments), the ALKO and wildtype controls did not differ in the blood-to-spinal cord transport of ^{125}I -leptin. In both wildtype and ALKO mice, the volume of distribution of ^{125}I -leptin was highest in the cervical cord, intermediate in the lumbar region, and lowest in the thoracic area. There was no difference in the influx rate (Fig. 6B). The half-life of ^{125}I -leptin in serum was the same as in the wildtype mice (Fig. 6C), indicating the absence of change in tissue distribution. Permeation of the co-administered paracellular permeability marker ^{131}I -albumin also was not different between the ALKO and wildtype

mice. This indicates that the general permeability of the BBB was unchanged by the ALKO mutation, and that injection of leptin did not cause additional changes in the vascular space.

Astrocytic activity in obese A^{vy} mice changes leptin distribution after its intracerebroventricular injection [31]. Thus, we further determined whether the ALKO mutation alters the fate of leptin once it crosses the BBB. Leptin degradation in the ALKO and wildtype mice did not differ over time in brain homogenates (Fig. 7).

Discussion

Cell-type specific actions of leptin in the CNS reflect the presence of a multi-component regulatory system to fine-tune neuroendocrine control. An early goal was to determine the roles of endothelial ObR and astrocytic ObR in leptin transport across the BBB. We felt this was feasible because neuronal specific LR knockout mice had already been generated, and their metabolic phenotype of obesity and diabetes fully characterized [27].

There were several technical challenges in the study. The main limitation was the nature of the LR-floxed mice available. It would be best if the loxP sites were inserted in such a way that all leptin receptor subtypes were deleted by cre or flp recombinases, without production of a mutant membrane-bound receptor. However, the existing LR-floxed mice have the loxP sites flanking Exon 17, leading to the generation of a mutant receptor ObR-Δ17 [26], with a mature protein containing amino acid (aa) 22-878. The mutant receptor contains the same extracellular domain (aa22-839), transmembrane domain (aa840-860) with 3 aa residues afterwards, and the mutant sequence SLKHLISIFLPSMQNQ that does not have known signaling function (Fig. 8). Our recent in-vitro results showed that truncated LR isoforms maintain full capacity to mediate leptin endocytosis despite the absence of a cytoplasmic tail [34]. This led to the prediction that the endothelial specific LR mutant mice would have persistent transport functions, and this was confirmed by the transport assays. The ELKO mice showed an influx rate of leptin after intravenous delivery similar to that of the wildtype controls. The results support our notion that a tailless receptor can mediate leptin-induced endocytosis through recruitment of clathrin and dynamin microdomains without a cytoplasmic sorting signal [34].

The lack of change of the apparent influx rate of leptin from blood to the CNS was further confirmed by measurement of influx rates in different brain regions and the cervical, thoracic, and lumbar segments of the spinal cord. However, capillary depletion studies indicated that ELKO mice had a slightly faster influx in the cerebral cortex. In-situ brain perfusion in different batches of mice of older age and opposite sex confirmed a significant increase of leptin uptake. In-situ brain perfusion and multiple-time regression studies are complementary approaches, and the discrepancy between the two tests often suggests situations where blood-borne factors interfere with the apparent influx rate [32,33]. Changes in leptin peripheral binding interactions could have occurred in the ELKO mice.

Since ELKO mice have mutation of membrane-bound LR in all endothelial cells, including peripheral blood vessels, the in-situ brain perfusion approach also helps to address cerebral uptake more selectively. Increased uptake of leptin after delivery by in-situ brain perfusion indicates that the ELKO mutation has a facilitating role to increase leptin permeation across the BBB. This suggests that endothelial leptin signaling may reduce leptin transport. The beneficial effect of ELKO in increasing the availability of leptin in the brain is consistent with partial resistance of the mutant mice to diet-induced obesity (Pan W et al., unpublished data).

Similar to the ELKO mice that have an LR mutation involving peripheral vessels as well as cerebral microvascular endothelial cells, the ALKO mice have LR mutation in not only

astrocytes but also a small portion of neuronal progenitor cells of GFAP lineage. This is opposite to the issues raised by use of Tet-off or tamoxifen responsive systems that are often associated with incomplete recombination with inducible cre recombinase, so that not all astrocytes have deletion of the target gene [11,12]. However, embryonic knockout by use of GFAP-cre mice assured sufficient yield of mutant mice since it has been extremely difficult to obtain large numbers of inducible ALKO offspring in our ongoing experiments.

Astrocytic activity affects neuronal leptin uptake and signaling, as the astrocyte metabolic inhibitor fluorocitrate increases the amount of leptin reaching and activating neurons after intracerebroventricular injection [31]. However, in-vitro studies have shown that basal levels of LR in astrocytes do not affect leptin transport across the endothelial monolayer, although overexpression of ObRb or ObRe decreases it [18]. In the ALKO mice, we confirmed that leptin transport across the BBB was unchanged. This is consistent with the cellular findings. The half-life disappearance of leptin in blood was also unchanged by the ALKO mutation, and the degradation kinetics of blood-borne leptin in both serum and brain homogenate did not show significant change from the controls. Thus, basal activities of astrocytic leptin signaling may not play a major role in leptin transport across the BBB.

We measured leptin transport not only in different regions of the BBB, but also at the blood-spinal cord barrier. The spinal cord does not contain circumventricular organs and has a circadian rhythm of leptin transport [33]. A neurotrophic effect of leptin in the brain is well-established [13,14]. Though it is not clear whether leptin acts on reactive astrocytes to modulate the course of regeneration after spinal cord injury, there is no doubt that leptin reaches the spinal cord where it may act. Neither ELKO nor ALKO mice, however, showed changes in the influx rate of leptin across the blood-spinal cord barrier.

In summary, the newly generated knockout mice express a mutant leptin receptor without signaling properties in endothelial cells and astrocytes. ELKO is associated with a moderate increase of leptin uptake into brain parenchyma shown only by in-situ brain perfusion, whereas ALKO does not affect leptin transport from blood to brain or spinal cord in the basal state (Fig. 8). These mice should be valuable tools to determine the respective roles of endothelial and astrocytic leptin signaling in disease pathology, such as obesity, metabolic disorders, or other forms of neuroinflammation.

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Research Highlights

- Generation and characterization of ELKO and ALKO mice for the first time
- Illustrating that membrane-bound mutant receptors without signaling functions can mediate leptin transport across the BBB
- Leptin signaling may hinder its own transport across endothelial barrier
- Astrocytic leptin receptors do not play a crucial role in basal level leptin transport
- Implications in obesity, metabolic syndrome, and neuroinflammation

Fig. 1. DNA genotyping of the offspring of LR^{loxP/+}/Tie2^{cre/wt} and LR^{loxP/loxP} mice was performed for Tie2-cre (A) and LR-flox (B). The left lane shows molecular weight (bp). Lane 1: wildtype (+/+, +/+); lane 2: LR-flox (+/+, f/f); lane 3: LR-flox, Tie2(tek)-cre (cre/+, f/f), the true ELKO mouse; lane 4: LR-flox heterozygote, Tie2-cre (cre/+, f/+); lane 5: LR-flox with general Exon 17 deletion, Tie2-cre (cre/+, Δ17/Δ17).

Fig.2A

Fig.2B

Fig.2C

Fig. 2.

The effect of the ELKO mutation on permeability of ^{125}I -leptin across the BBB was tested in 3 m old mice by intravenous delivery of the tracer and multiple time-regression analysis ($n = 8/\text{group}$). (A) The influx rate and volume of distribution of ^{125}I -leptin in the brain did not differ significantly between the ELKO and wildtype mice 1 – 20 min after intravenous delivery. (B) The influx rate of ^{125}I -leptin in different brain and spinal cord regions did not differ between the ELKO and wildtype mice. (C) Cortical samples were subjected to the capillary depletion procedure, which showed that parenchymal uptake was significantly ($p < 0.005$) higher than that of the enriched microvessels in both ELKO and wildtype mice; however, there was no significant increase of influx rate of leptin to brain parenchyma in the ELKO mice.

Fig. 3.

The effect of the ELKO mutation on brain uptake of ^{125}I -leptin was determined by 5 min of in-situ brain perfusion, followed by capillary depletion. The ELKO mice ($n = 5$) showed a higher uptake in both parenchymal and capillary fractions than the wildtype controls ($n = 6$).

*: $p < 0.05$.

Fig. 4. Genotyping of ALKO mice. (A) DNA genotyping from tail samples of a representative litter of mice that contains wildtype (floxed LR, wildtype LR, wildtype GFAP-cre) and ALKO (LR-Δ17, GFAP-cre) PCR products. The asterisk (*) represents the mouse with GFAP-cre and LR-flox heterozygote, which was not used in the experiments. (B) PCR from cultured primary astrocytes from different brain regions in the wildtype and ALKO mice. In the wildtype cortex (lane 1) and hippocampus (lane 2), wildtype GFAP-cre, floxed LR, and

wildtype LR products were present. In the ALKO cortex (lane 3) and hippocampus (lane 4), GFAP-cre and LR- $\Delta 17$ were present.

Fig. 5. Immunohistochemistry verifying the successful and specific deletion of ObRb in the ALKO mice. Absence of ObRb immunofluorescence was seen in $\alpha 1$ tanycytes lining the third ventricle adjacent to the dorsal medial hypothalamus (arrows). In the wildtype mouse, confocal microscopy showed co-localization of ObRb and GFAP in this region. Neuronal ObRb was unchanged by ALKO mutation. The GFAP(+) tanycyte processes showed a tortuous and abbreviated morphology in the ALKO mice, indicative of mild astrogliosis. Scale bar: 40 μ m.

Fig.6A

Fig.6B

Fig.6C

Fig. 6.

BBB transport of leptin in the ALKO mice ($n = 8/\text{group}$). (A) In whole brain, the ALKO and wildtype groups did not differ in the rate of influx or volume of distribution of ^{125}I -leptin. (B) In different regions of the spinal cord (cervical, thoracic, and lumbar segments), the ALKO and wildtype controls did not differ in the blood-to-spinal cord transport of ^{125}I -leptin. (C) The disappearance of ^{125}I -leptin in serum followed a 2-phase exponential decay. There was no difference between the wildtype and ALKO groups.

Fig. 7.

Lack of effect of ALKO mutation on degradation patterns of leptin in serum and brain of mice at different times after intravenous delivery of ^{125}I -leptin. Acid precipitation showed that ^{125}I -leptin remained mostly intact within the first 7 min, but had a linear degradation over time. There was no difference in the % intact leptin in the brain homogenates of the wildtype and ALKO mice ($n = 8/\text{group}$).

Fig. 8.

Diagram of the structure of mutant ObR in the ALKO mice. (A) The LR-flox mice contain two loxP sites flanking exon 17, and recombination with human GFAP-driven Cre-recombinase gene leads to excision of exon 17 and a premature stop codon at aa878. The resultant mutant LR-Δ17 does not contain box 1 shared by the short-forms of membrane-bound LR (a, c, d) or box 2 and 3 unique to ObRb. (B) Features of ELKO mice. (C) Features of ALKO mice. DNA genotyping for ELKO mice. Lane 1: wildtype (+/+, +/+); lane 2: LR-flox (+/+, f/f); lane 3: LR-flox, Tie2(tek)-cre (cre/+, f/f), the true ELKO mouse; lane 4: LR-flox heterozygote, Tie2-cre (cre/+, f/+); lane 5: LR-flox with general Exon 17 deletion, Tie2-cre (cre/+, Δ17/Δ17).

Table 1

Primers used for DNA genotyping from mouse tail

	Forward primer	Reverse primer	Size of amplicon
Tie2-Cre recombinase transgene *	<u>oIMR0042</u> 5'-CTAGGCCACAGAATTGAAAGATCT-3' <u>oIMR1084</u> 5'-GCGGTCTGGCAGTAAAACTATC-3'	<u>oIMR0043</u> 5'-GTAGGTGGAAATTCTAGCATCATCC-3' <u>oIMR1085</u> 5'-GTGAAACAGCATTGCTGTCACTT-3'	Wild type: 324 bp Transgene: 100 bp
GFAP-Cre recombinase transgene *	<u>oIMR1900</u> 5'-ACTCCTTCATAAAGCCCT-3'	<u>oIMR1901</u> 5'-ATCACTCGTTGCATCGACCG-3'	Wild type: 700 bp Transgene: 190 bp
LR-flox **	<u>103</u> 5'-TGAGTTCCTCATGATTCTGG-3' <u>104</u> 5'-CAGCCGACCAATGCTTATT-3'	<u>105</u> 5'-ACAGGCTTGAGAACATGAACAC-3'	Wild type: 289 bp Floxed: 339 bp Δ17: 208 bp

* sequences supplied by the Jackson Laboratory

** sequences from McMinn et al. [26]