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MOLECULAR METABOLISM

Phenotypic effects of an induced mutation of the ObRa isoform of the leptin receptor*

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ABSTRACT

Leptin receptors play critical roles in mediating leptin's pleiotropic effects on mammalian physiology. To date, six splice variants of the leptin receptor gene have been identified [1–3]. These splice variants have identical extracellular leptin binding motifs but different intracellular C termini. The finding that mutations specifically ablating the function of ObRb cause obesity has established a critical role for this isoform in leptin signaling [1,7]. ObRa is the most abundant splicing isoform with a broad tissue distribution [5], and it has been proposed to play roles in regulating leptin bioavailability, CSF (cerebrospinal fluid) transport and function by forming heterodimers with ObRb and also activating signal transduction via JAK2 in-vitro [5–10]. To assess the in-vivo role of ObRa, we generated an ObRa KO mouse by deleting the ObRa-specific exon 19a. Homozygous mutant mice breed normally and are indistinguishable from wild-type mice on regular chow diet, but show a slightly increased basal plasma leptin, a slight improvement of their GTT and a slightly reduced response to systemic leptin administration. These mice also show a modest but statistically significant increase in weight when placed on a high fat diet with a slightly reduced CSF/plasma ratio of leptin. These data suggest that ObRa plays a role in mediating some of leptin's effects but that the phenotypic consequences are modest compared to a deletion of ObRb.

Keywords ObRa; Leptin; Obesity; Leptin resistance; Glucose homeostasis; Blood brain barrier

1. INTRODUCTION

Leptin is a 16 kDa peptide hormone produced by adipocytes that regulates food intake, body weight and many (possibly all) other physiologic systems [1,11–15]. Leptin's primary site of action is in the central nervous system, in particular the hypothalamus where a dense population of neurons expressing the leptin receptor reside [16–19].

Leptin receptors are members of the gp130 family of cytokine receptors, which are single trans-membrane proteins and rely on enzymatic activities from other proteins to achieve signal transduction. The initial discovery of the leptin receptor gene revealed two principal splice variants known as ObRa and ObRb [5,4]. Both forms have identical extracellular leptin-binding domains at the N-terminus, but different intracellular C-termini, each with distinct signal transduction capability. The ObRb intracellular segment is 302 amino-acids long with a JAK2 binding site (Box1) and three tyrosine phosphorylation motifs. When leptin binds, conformational changes of ObRb lead to phosphorylation of JAK2. JAK2 further auto-phosphorylates and fully activated JAK2 leads to phosphorylation of all three tyrosine residues, each of which recruits a unique set of signaling partners, including STAT3, STAT5 and SHP2/SOCS3/ERK. A series of site-specific mutagenesis studies showed elegantly that these tyrosine phosphorylation motifs play differential but overlapping roles in mediating leptin's physiological effects in vivo [20–22].

The functional importance of ObRb has been informed by analyses of mice that carry mutations in the leptin receptor gene (the *db* locus). C57BL/6J db^{ks}/db^{ks} mice have a mutation that alters splicing such that ObRa is expressed in place of ObRb with no alternations to the other isoforms [1]. The phenotype of these animals is identical to that of db^{3J}/db^{3J} and db^{pas}/db^{pas} mice which carry null mutations of the leptin receptor gene and do not produce any of the leptin receptor isoforms [18,23,24]. These data indicate that ObRb, which has a narrow tissue distribution in the hypothalamus and hematopoietic cells, is the primary functional isoform. Consistent with ObRb's tissue distribution, further studies using brain-specific deletion and genetic rescue experiments have shown that the central nervous system is the primary site of action for leptin's weight reducing effects [24–26].

ObRa was the first splice variant to be identified and is expressed in the choroid plexus and many other tissues. ObRa has a short intracellular loop (34aa) which contains the JAK2 binding site but does not include any of the three tyrosine phosphorylation sites that are necessary for signal transduction by this class of receptors, at least in the absence of a hetero-dimeric binding partner [27,28]. Although the afore-mentioned studies firmly established the role of ObRb in leptin action in the brain, the data did not exclude the

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possibility that ObRa is also required for leptin to exert some or all of its effects. Indeed prior studies using transfected cells have shown that ObRa can phosphorylate JAK2 and activate IRS-1 and ERK [29] and that ObRa also leads to receptor-mediated internalization of leptin and degradation by lysosomes [30,31].

Other studies have shown that leptin enters the brain through a saturable mechanism [32,33]. The high levels of ObRa in choroid plexus and brain micro-vessels, two major components of the blood–brain barrier, have also suggested that ObRa could play a role in leptin transport into the brain [4–6,34,35]. It has thus been hypothesized that ObRa may function as a high-affinity leptin transporter at the blood–brain barrier.

In order to study ObRa and its potential role in leptin transport and function in vivo, we generated an ObRa knock-out mouse by deleting the ObRa-specific exon 19a, using conventional Cre-mediated homologous recombination, and report the metabolic phenotypes of these mice here.

2. MATERIALS AND METHODS

2.1. Animals

In order to specifically delete the ObRa-specific exon (19a), two *cis* loxP sites (see Supplementary Figure 1 for details) were inserted upstream and downstream of the coding region of 19a. Simultaneously, a PGK-Neo cassette (flanked by two *cis* FRT sites) was inserted upstream of 19a, to facilitate the selection of positively targeted ES cell clones. A 486 bp sequence (Probe 1) upstream from the PGK-Neo insertion site was used to generate the probe to distinguish the wild-type (wt) and the mutant (mu) allele by Southern blot. The primer sequences for PCR amplifying the probe sequence are: Forward 5′-TCATCTCTTCAGTTCCTTAATTCC and Reverse 5′-GGAAGACAGGGCATCAAATGAG. The AfI II site immediately downstream from 19a was mutated from CTTAAG to CTTAATAAG in the mutant allele, so that AfI II digestion would yield a hybridization band of 16.55 kb for the mutant allele and a 7.24 kb band for the wild-type allele. A third AfI II site 7.33 kb downstream from the second one was not shown. Due to the reduced transfer efficiency of the significantly larger mutant band, an EcoRV site located 1 kb downstream from the mutated AfI II site was used in addition to AfI II, which reduced the size of the mutant band to 10.27 kb supplementary material Table 1. DNA sequencing was used to verify the correct insertion and orientation of the loxP and FRT sites (data not shown).

Following the establishment of the correctly targeted founder line, mice carrying the mutant allele were crossed to mice carrying the *Saccharomyces cerevisiae* FLP1 recombinase gene ("the Flipper mouse", 129S4/SvJaeSor-Gt(ROSA)26Sortm1(FLP1)Dym/J, stock# 003946, The Jackson Laboratory) to delete the PGK-Neo cassette. Progenies from this cross (designated as " × Flipper" in Table 1) were crossed to Ella-Cre mice (B6.FVB-Tg(Ella-cre)C5379Lmgd/J, stock # 003724,The Jackson Laboratory) to delete 19a. Progenies from this cross were designated as "(× Flipper) × Ella-Cre". Southern blots were used to identify mutant mice at all steps up to this point (Table 1). Four primers (F1: 5′-TGGTCGAAGAAAAGGAAGAGATTTG; R1: 5′-TCCCAAGTGCTGGGATTAAAG GC, F2: 5′-ACCATGAAAAGACAAGGGGTTAGAG and R2: 5′-AAAATGCA-GAGTCCATGAATATCAAC) were designed to amplify the corresponding sequences in the "(× Flipper) × Ella-Cre" progenies (both wild-type and mutant animals) by PCR. Sequencing results (not shown) confirmed the insertion and direction of the FRT and loxP sites, the correct deletion of 19a and the ablation of the second AfI II site. At this point, we designated the mutant allele from the "(× Flipper) × Ella-Cre" progenies as the ObRa KO allele. Heterozygous mice carrying the ObRa KO allele were intercrossed to generate homozygous ObRa KO and WT animals used in this study. Primer pairs (F1 + R2 and F2 + R2) were used to perform PCR genotyping in later generations (see supplementary material Table 2 for details on KO and WT amplicon sizes).

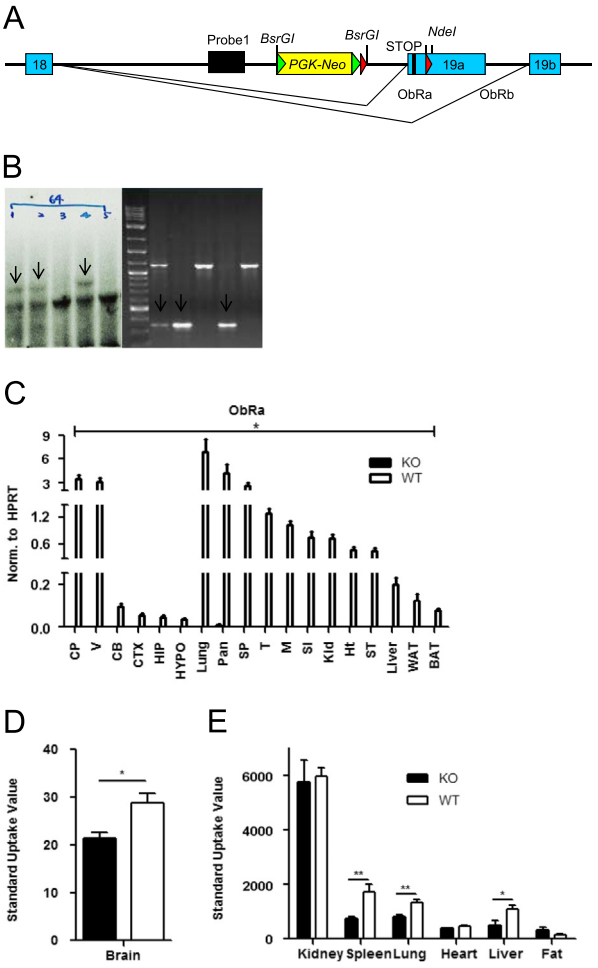

Figure 1: Generating the ObRa knock-out mice. ObRa KO mice were generated using standard protocol through cre-mediated homologous recombination. ObRa expression in homozygous mutant mice was measured by transcript-specific Taqman real-time qPCR. Leptin-bioditribution essay was used to evaluate the effect of ObRa KO on leptin binding to target tissues. (A) Diagram illustrating the molecular design of the knock-out allele of the ObRa genomic sequence (see more details in supplementary Figure 1). Briefly, FRT site (green triangle)-flanked PGK-Neomycin cassette was inserted upstream of the ObRa-specific exon, 19a. A pair of LoxP sites (red triangles) were inserted upstream and downstream of the coding region of 19a. The stop codon of 19a was indicated by a thick black line. The mutant allele was identified by Southern blot using a probe ("Probe 1", black rectangle) that hybridized to a 486 bp region in the wild-type genomic sequence upstream of the PGK-Neo insertion site. Exon 18 and the ObRb-specific exon, 19b, remained intact. (B) Southern blot and PCR genotyping results identifying the wt and mutant alleles in the "(× Flipper) × Ella-cre" progenies. Three out of five pups were shown to be heterozygous mutant by Southern blot after AfI II + EcoRV digestion (left panel; arrows indicated the mutant band). PCR gel (right panel) showed simultaneous amplification of the wt and mutant alleles by F1 + R2 and identified the same three heterozygous mutant mice out of the five pups. *Note*: due to the size difference, the wt band was sometimes not amplified as shown for pups #2 and 4. Thus, F2 + R2 were routinely used to confirm the presence of the wt allele thereafter (data not shown). (C) Expression of ObRa was completely abolishment in ObRa KO vs. WT mice ($n = 5$ each). CP (choroid plexus), V (brain micro-vessels), CB (cerebellum), CTX (cortex), HIP (hippocampus), HYPO (hypothalamus), Lung, Pan (pancreas), SP (spleen), T (testis), M (skeletal muscle), SI (small intestine), Kid (Kidney), Ht (heart), ST (stomach), Liver, WAT (white adipose tissue) and BAT (brown adipose tissue) were tested. *: $p < 0.05$ for all paired comparisons in each tissue. (D and E) I^{125}-labeled leptin was injected to live ObRa KO and WT mice ($n = 6$ each) through the tail-vein. Whole brain homogenate (21.46 ± 1.22 vs. 28.86 ± 1.91, $p < 0.05$), spleen (749.87 ± 67.66 vs. 1729.80 ± 260.86, $p < 0.01$), lung (826.62 ± 38.27 vs. 1325.68 ± 129.08, $p < 0.01$) and liver (504.81 ± 174.90 vs. 1082.06 ± 147.74, $p < 0.05$) showed significantly reduced leptin binding in ObRa KO vs. WT mice.

Generation of correctly targeted ES cell clones and chimeric founder strains were carried out at the Gene Targeting and the Transgenic Services at The Rockefeller University.

All animal experiments were in compliance with regulations at the Rockefeller University Institutional Animals Care and Use Committee (IACUC, protocol #11402) and NIH. Animals were group housed at room temperatures ranging from 68 to 73 °F unless otherwise stated in the method of the experiment and subjected to 12/12 light/dark cycles.

2.2. Quantitation of ObRa, ObRb, ObRc, ObRe and ObR total expression

Choroid Plexus (CP), micro-vessel (V), hypothalamus (Hypo), hippocampus (Hip), cerebellum (CB), cortex (CTX), lung, pancreas (Pan), spleen (SP), testis (T), quadriceps (M), small intestine (SI), kidney (Kid), heart (Ht), stomach (ST), liver, epididymal white adipose tissue (WAT) and brown adipose tissue (BAT) were removed from ObRa KO and WT adult male littermates fed ad libitum ($n=5$ each). The preparation of brain micro-vessels was carried out as previously described [34]. Total RNA was extracted using Trizol (Invitrogen), purified using RNeasy Micro Kit (Qiagen), and reverse transcribed using QuantiTect RT Kit (Qiagen). Real-time qPCR was performed using transcript-specific Taqman probe sets from ABI: Mm01262070_m1 (ObRa), Mm01265583_m1 (ObRb) and Mm00440181_m1 (ObR Total), and IDT DNA (custom-made): ObRc (forward primer 5′-TCCTACTGCTCGGAACACTGTTAA; reverse primer 5′-CAAACCCAGCTTTTGAGAAAGAG; probe 5′-AAGCCTGTTCATCCTT) and ObRe (forward primer 5′-CAGCGCACACTGTTACAGTTCTG; reverse primer 5′-TGATTTATTATCAGAATTGCCAGTCTACAG; probe 5′-GTATGTGTACTG-TACTTTTCATGG). All values were normalized to that of HPRT. Due to reduced quality of pancreatic total RNA preparation, Taqman results for ObRc, e and Total were excluded from Figure 6C–E.

2.3. Biodistribution of ^{125}I-leptin

This assay was carried out as previously described [36]. Briefly, 8–12 KBq of ^{125}I-leptin (PerkinElmer, New England Nuclear) was injected into the tail vein of adult ObRa KO and WT mice ($n=6$ each), fed ad lib. The animals were sacrificed 15 min post-injection. The indicated tissues were removed and weighed before the radioactivity was counted with a gamma counter. Standard uptake value on the *Y*-axis is defined as the % injected dose/gram of tissue, divided by % injected dose per mg of blood. This analysis is used to correct for the varying levels of leptin in the blood in the different animal groups.

2.4. Indirect calorimetry

Fourteen and 28 week old male ObRa KO and WT mice ($n=6$ each) were acclaimed to the experimental chambers overnight prior to data collection using the Oxymax Lab Animal Monitoring System (Columbus Instruments). VO_2, VCO_2, RER and HEAT were averaged over 24 h.

2.5. Plasma leptin and insulin measurement

Food was taken away from 10 am to 2 pm before retro-orbital bleeding was performed to collect blood using EDTA-coated capillaries (Drummond Scientific). Blood was kept on ice and centrifuged at 4000 rpm, 4 °C for 15 min. Serum was transferred to a new tube and kept at −20 °C until measurement. Leptin and insulin were measured according to manufacturer's instructions: Mouse Leptin Quantikine Elisa Kit (R&D), Mouse Insulin Ultrasensitive EIA (ALPCO).

2.6. Fasting glucose and glucose tolerance test

Mice were fasted overnight (14–16 h) before the onset of glucose tolerance test. Ten microliter of 10% glucose in 0.9% NaCl was intraperitoneally injected per gram of body weight. Blood was collected by tail snipping immediately before the injection and at 15, 30, 45, 60, 90 and 120 min post-injection. Blood glucose was measured by Breeze2 blood glucose meter (Bayer Healthcare LLC).

2.7. High-fat diet treatment

ObRa KO ($n=16$) and WT ($n=14$) male littermates were subjected to high fat diet (HFD, 60 kcal% fat, Research Diet, D12492) starting at 6 weeks of age. Body weight was measured weekly. After 26 weeks of HFD treatment, body composition was measured by Dual Energy X-ray Absorptiometry (Lunar PIXImus2) and GTT was performed. Plasma and CSF were collected for leptin measurement (Mouse Leptin Elisa Kit, Crystal Chem, 90030). The collection of CSF was carried out as previously described [37].

2.8. Leptin treatment

ObRa KO ($n=22$) and WT ($n=10$) male littermates were given recombinant mouse leptin (800 ng/h, Amylin Pharmaceuticals) via subcutaneous osmotic pumps (Alzet, model 2002) at 16 weeks of age. Body weight and food intake were measured every other day. Pumps were removed after 14 days of infusion. BW and FI were followed up for another 14 days. Changes in BW were expressed as percentage of BW at day 0 respective for the two phases.

2.9. STAT3 activation

ObRa KO and WT male littermates were given acute 1 μg/g BW leptin or PBS through intra-peritoneal injection or acute 20 ng leptin in 1 μl PBS through stereotactic intra-cerebra-ventricular injection. Animals were sacrificed 45 min after injection and STAT3 activation in the hypothalamus was essayed by Western blot (WB, for which hippocampus was also essayed) or immunohistochemistry (IHC) for phosphorylated and total STAT3 (mouse anti-STAT3 clone 124H6 and rabbit anti-pSTAT3 (Tyr705) clone D3A7, Cell Signaling). For WB, leptin-induced pSTAT3 signal intensity was expressed as fold increase in comparison to pSTAT3 signal intensity under PBS treatment. For IHC, fluorescence-labeled

	14 weeks					28 weeks				
	KO		WT			KO		WT		
	Mean ± SEM	*n*	Mean ± SEM	*n*	*p*	Mean ± SEM	*n*	Mean ± SEM	*n*	*p*
BW (g)	27.15 ± 0.69	13	27.62 ± 0.56	6	> 0.05	30.96 ± 1.22	13	31.56 ± 1.57	6	> 0.05
FI (g)	5.18 ± 0.23	24	4.43 ± 0.12	10	> 0.05	5.17 ± 0.19	24	5.1 ± 0.16	10	> 0.05
Lean mass (g)	20.16 ± 0.34	37	20.18 ± 0.41	16	> 0.05	21.88 ± 0.65	13	22.33 ± 0.49	6	> 0.05
% Fat	10.06 ± 0.32	37	10.26 ± 0.54	16	> 0.05	17.48 ± 1.82	13	17.97 ± 3.12	6	> 0.05
VO_2 (ml/kg/h)	3844.18 ± 203.72	12	3566.75 ± 148.13	6	> 0.05	4181.39 ± 193.16	12	3977.82 ± 159.00	6	> 0.05
VCO_2 (ml/kg/h)	3932.04 ± 191.54	12	3600.43 ± 154.84	6	> 0.05	4441.03 ± 210.22	12	4121.27 ± 192.56	6	> 0.05
RER	1.03 ± 0.02	12	1.01 ± 0.02	6	> 0.05	1.07 ± 0.01	12	1.04 ± 0.02	6	> 0.05
HEAT (kcal/h)	0.55 ± 0.02	12	0.52 ± 0.02	6	> 0.05	0.60 ± 0.02	12	0.58 ± 0.02	6	> 0.05

Table 1: Basal metabolic parameters.

brain sections were imaged using Zeiss LSM 510 laser scanning confocal microscope (The Biological Imaging Research Center at The Rockefeller University). All parameters (pinhole, stack step size, zoom, laser power, excitation light intensity, and gain) were kept the same for all specimens. Brightness and contrast were slightly adjusted for figure presentation. No nonlinear adjustment was carried out. Fluorescence intensity calculation and cell count were done using semi-automated functions in Imaris (all parameters kept the same, Bitplane Scientific Software).

2.10. Statistical analysis

Data were expressed as mean $\pm$ SEM (standard error of the mean). Student's *t* tests were used to evaluate the significance of difference of the mean between ObRa KO and WT animals. $p < 0.05$ represents statistical significance.

3. RESULTS

3.1. Generation of an ObRa knock-out mouse

ObRa and ObRb share the same first 18 exons of the leptin receptor genomic sequence. Alternative splicing of exon 18 to the two alternative 3′ exons (19a and 19b) of the leptin receptor gene generates ObRa and ObRb respectively. To generate an ObRa-specific knockout, a pair of loxP sites was inserted flanking the coding region of 19a (Figure 1A and Supplementary Figure 1). A Neo cassette, flanked by a pair of FRT sites was inserted immediately upstream of the first loxP site, to facilitate the selection of positively targeted ES cell clones. Chimeric founders carrying the correctly integrated targeting vector and germ-line transmission of the mutant allele were identified by Southern blot (Figure 1B and Supplementary Figure 1). The Neo cassette was removed by crossing to the "Flipper" mouse. Mutant progenies from this cross were crossed to Ella-Cre mouse to remove the ObRa-specific exon 19a, with subsequent progeny from this cross backcrossed to C57BL/6J and used for generating the wild-type (WT) and homozygous mutant (KO) mice.

ObRa-specific Taqman real-time qPCR was performed on choroid plexus, brain micro-vessels, cerebellum, cortex, hippocampus, hypothalamus, lung, pancreas, spleen, testis, skeletal muscle, small intestine, kidney, heart, stomach, liver, white adipose tissue and brown adipose tissue from ObRa KO and WT mice (Figure 1C). ObRa mRNA was not detected in the KO mice for all tissues tested ($p < 0.05$ for all paired comparisons). Consistent with previous studies, choroid plexus and brain micro-vessels showed the highest level of ObRa expression in the brain in WT mice, while ObRa mRNA was broadly distributed in many other central and peripheral tissues.

Previous studies using radio-labeled leptin showed that leptin binding in the brain is highly enriched in the choroid plexus [5,38]. We reasoned based on this correlation between ObRa expression and leptin binding that if ObRa binds leptin substantially in vivo, tissues with high levels of ObRa expression in WT mice would show reduced leptin binding in KO mice. In order to test this hypothesis, ^{125}I-labeled leptin was injected into the tail veins of ObRa KO and WT mice. Mice were sacrificed 15 min post-injection and tissues were dissected and quantitated for radioactivity after correcting for the total amount of radioactivity in the blood. As shown (Figure 1D and E), most ObRa-expressing tissues indeed showed a significant reduction of leptin binding in KO vs. WT mice: brain (21.46 ± 1.22 vs. 28.86 ± 1.91, $p < 0.05$), lung (826.62 ± 38.27 vs. 1325.68 ± 129.08, $p < 0.01$), spleen (749.87 ± 67.66 vs. 1729.80 ± 260.86, $p < 0.01$), and liver (504.81 ± 174.90 vs. 1082.06 ± 147.74, $p < 0.05$), while no significant different was observed in kidney (5742.19 ± 814.32 vs. 5940.82 ± 346.73, $p > 0.05$), heart (348.83 ± 6.56 vs. 455.23 ± 45.58, $p > 0.05$) and fat (327.23 ± 109.72 vs. 161.70 ± 17.46, $p > 0.05$). These data confirmed that ObRa binds leptin in vivo.

3.2. Phenotypic assessment of ObRa KO mice on regular chow diet

ObRa KO mice were fertile and their phenotype was indistinguishable from WT mice when assayed for a battery of metabolic parameters (Figure 2A and B and Table 1): body weight, food intake, fat mass, lean mass, fat%, VO_2, VCO_2, heat production and RER.

However, 14 but not 28 week-old ObRa KO mice showed an increase in plasma leptin, the magnitude of which was small but statistically significant (Figure 2C, 14-week: 2.26 ± 0.45 vs. 0.98 ± 0.14 ng/ml, $p < 0.01$; 28-week: 10.79 ± 3.55 vs. 11.56 ± 4.57 ng/ml, $p > 0.05$). Plasma insulin was similar at both time-points (Figure 2D, 14-week: 0.47 ± 0.05 vs. 0.50 ± 0.07 ng/ml, $p > 0.05$; 28-week: 1.11 ± 0.27 vs. 1.02 ± 0.35 ng/ml, $p > 0.05$). ObRa KO mice had a small but significantly reduced fasting blood glucose at both ages (Figure 2E, 14-week: 64.4 ± 2.6 vs. 76.5 ± 7.0 mg/dL $p < 0.05$; 28-week: 66.5 ± 4.21 vs. 87.3 ± 10.8 mg/dL, $p < 0.05$) with an improved GTT at 28 weeks of age (Figure 2G, 30, 90, 120 min post-injection: 247.8 ± 10.5 vs. 305.3 ± 23.6 $p < 0.05$, 130.6 ± 6.4 vs. 173.2 ± 5.4 $p < 0.01$, 122.8 ± 6.0 vs. 160.7 ± 16.4 $p < 0.05$). The area under the curve calculation for the GTT was also significantly lower in KO vs. WT at this time-point (Figure 2H, 1131 ± 34.29 vs. 1340 ± 74.87 mg/dL*120 min, $p < 0.01$).

3.3. Phenotypic assessment of ObRa KO mice on a high fat diet

Starting at 6 weeks of age, ObRa KO ($n=16$) and WT ($n=14$) male littermates were fed a 60% high-fat diet. ObRa KO mice gained weight similarly to WT mice for the first 12 weeks (Figure 3A, $p > 0.05$ for all paired comparisons at each time-point; for example, week 0: 18.62 ± 0.26 vs. 19.26 ± 0.51 g; week 12: 50.09 ± 0.64 vs. 47.55 ± 1.59 g). During weeks 13–22 of HFD (ages 19–28 weeks), ObRa KO males showed a small but statistically significant increase in weight compared to WT ($p < 0.05$ at each time point during weeks 13–22; for example, week 16: 53.85 ± 0.75 vs. 49.39 ± 1.83 g). However, starting at week 23 of the HFD treatment (29 weeks old), the weight of both groups became similar again ($p > 0.05$ at each time point after week 22; for example, week 23: 58.31 ± 0.97 vs. 54.76 ± 1.90 g). Despite this, ObRa KO did show a small but significantly increased fat mass vs. WT by both absolute mass (Figure 3B, 25.0 ± 0.7 vs. 22.4 ± 0.6 g, $p < 0.05$) and percentage of fat relative to body weight (43.1 ± 0.6 vs. 40.0 ± 0.6%, $p < 0.01$) by the end of the treatment (week 26), when mice were 32 weeks old. This is in contrast to similar fat mass (2.2 ± 0.2 vs. 2.6 ± 0.2 g, $p > 0.05$) and percentage of fat (10.6 ± 0.5 vs. 12.0 ± 0.8%, $p > 0.05$) between the two genotypes observed at 28 weeks of age on a standard chow diet (Table 1). In further contrast to chow-fed animals, fasting glucose (Figure 3D, time 0, 138.2 ± 3.7 vs. 135.9 ± 5.5 mg/dL, $p > 0.05$; compare Figure 2E) and GTT (Figure 3D, $p > 0.05$ at all time-points; compare Figure 2G) were not different in ObRa KO and WT mice on a high fat diet. AUC calculation showed no significant difference either (data not shown; compare Figure 3H).

Consistent with the increased fat mass, plasma leptin levels were significantly increased in ObRa KO vs. WT mice (Figure 3C, 130.54 ± 16.50 vs. 77.06 ± 11.3 ng/ml, $p < 0.01$). This is in contrast to undistinguishable plasma leptin levels in 28-week old mice fed on regular chow (Figure 2B, 10.79 ± 3.55 vs. 11.56 ± 4.57 ng/ml, $p > 0.05$). We next tested whether this highly

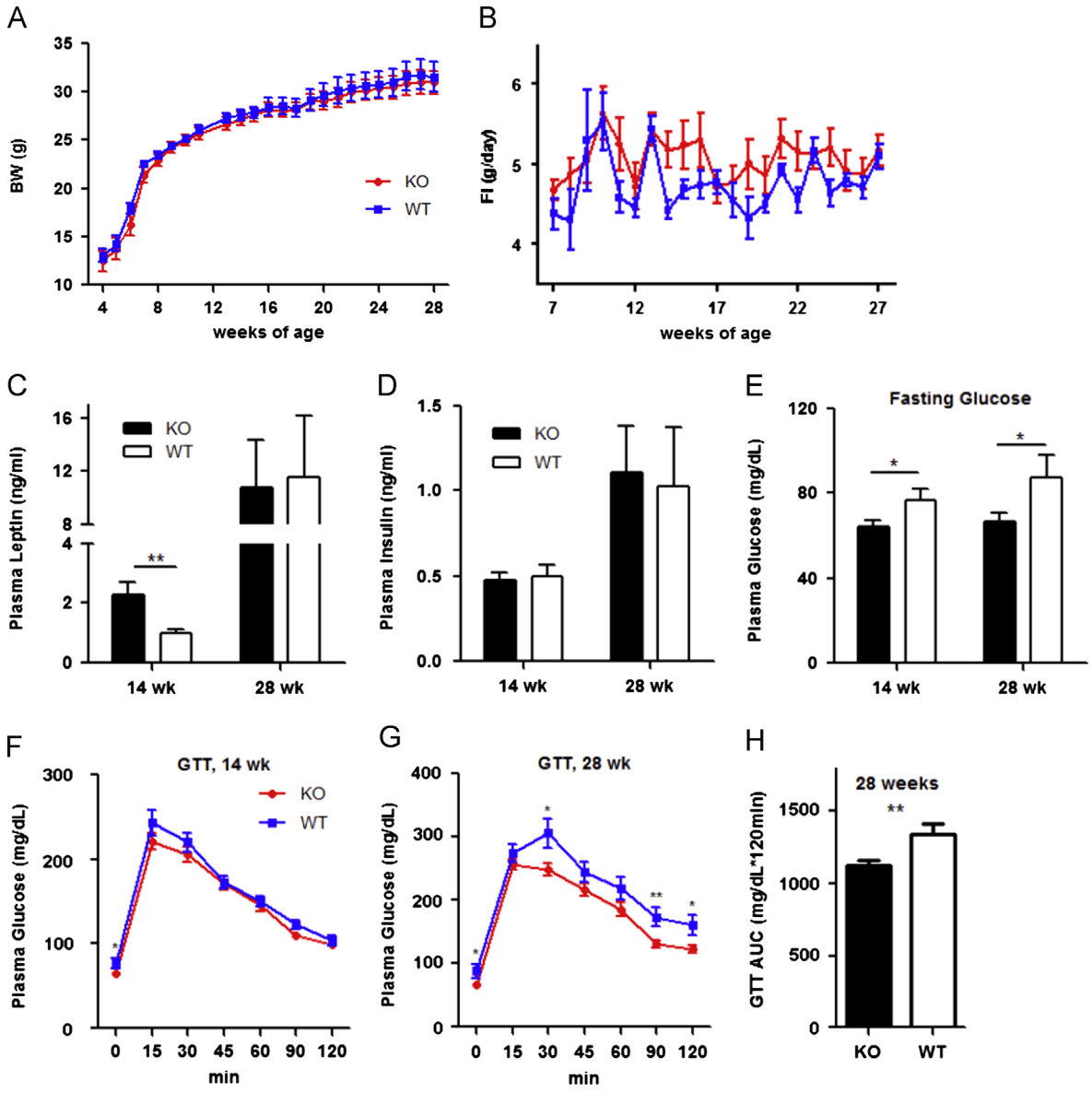

Figure 2: Phenotypic assessment of ObRa KO mice on regular chow diet. ObRa KO and WT male littermates were fed with regular chow (10% fat). Body weight, food intake, plasma leptin, insulin, fasting blood glucose and GTT were measured. (A and B) Body weight and food intake in littermates of ObRa KO ($n=13$ for BW and $n=24$ for FI) and WT ($n=6$ for BW and $n=10$ for FI) male mice showed no significant difference ($p>0.05$ at all time-points). (C) Plasma leptin was elevated in 14 week old male ObRa KO ($n=23$) vs. WT ($n=10$) mice (2.26 ± 0.45 vs. 0.98 ± 0.14 ng/ml, $p<0.01$) but similar in 28 week old mice (10.79 ± 3.55 vs. 11.56 ± 4.57 ng/ml, $p>0.05$). (D) Plasma insulin levels showed no significant difference in male ObRa KO ($n=24$ and 13) and WT littermates ($n=10$ and 6): 14-week: 0.47 ± 0.05 vs. 0.50 ± 0.07 ng/ml, $p>0.05$; 28-week: 1.11 ± 0.27 vs. 1.02 ± 0.35 ng/ml, $p>0.05$. (E) Fasting glucose was significantly reduced in ObRa KO mice ($n=35$ and 13) vs. WT mice ($n=16$ and 6) at both 14 week (64.4 ± 2.6 vs. 76.5 ± 7.0 mg/dL $p<0.05$) and 28 week of age (66.5 ± 4.21 vs. 87.3 ± 10.8 mg/dL, $p<0.05$). (F and G) Glucose tolerance tests showed no significant difference in 14 week old ObRa KO vs. WT littermates ($n=35$ and 16 respectively, $p>0.05$ for paired comparison at each time-point) but significant improvement in 28 week old male littermates ($n=13$ for KO and 6 for WT; 30, 90, 120 min post-injection: 247.8 ± 10.5 vs. 305.3 ± 23.6 $p<0.05$, 130.6 ± 6.4 vs. 173.2 ± 5.4 $p<0.01$, 122.8 ± 6.0 vs. 160.7 ± 16.4 $p<0.05$). (H) Area Under the Curve (AUC) calculation for the GTT at 28 weeks of age showed that ObRa KO had significantly better GTT performance than WT (1131 ± 34.29 vs. 1340 ± 74.87 mg/dL*120 min, $p<0.01$).

elevated plasma leptin was also associated with an elevated CSF leptin. In fact, we found that CSF leptin appeared lower in ObRa KO mice (despite the higher plasma leptin levels), but the difference was not significant (0.49 ± 0.10 vs. 0.88 ± 0.36 ng/ml, $p>0.05$). Nonetheless, the ratio of CSF to plasma leptin was significantly lower in ObRa KO than WT (Figure 3C, 0.45 ± 0.10 vs. 1.51 ± 0.58, $p<0.05$).

3.4. Leptin sensitivity in ObRa KO mice

ObRa KO ($n=22$) and WT ($n=10$) littermate males were treated with a subcutaneous infusion of leptin at 800 ng/h for 15 days. As shown in Figure 4A, the body weight of KO and WT males started to diverge on day 3 and remained separated for the remainder of the treatment (day 0: KO 28.18 ± 0.38 g vs. WT 27.27 ± 0.59, $p>0.05$; day 3: 27.28 ± 0.43 vs. 25.77 ± 0.61, $p>0.05$; day 9: 26.61 ± 0.47 vs. 25.13 ± 0.68, $p>0.05$). Although the separation appeared obvious, the difference between the two groups did not reach statistical significance. We tested whether this was due to high intra-group variation by expressing the value of body weight reduction as a percentage of the pre-treatment value. As shown in Figure 4B, KO showed significantly reduced body weight reduction during the course of leptin treatment (day 3: -3.4 ± 0.4 vs. -5.5 ± 0.7%, $p<0.01$; day

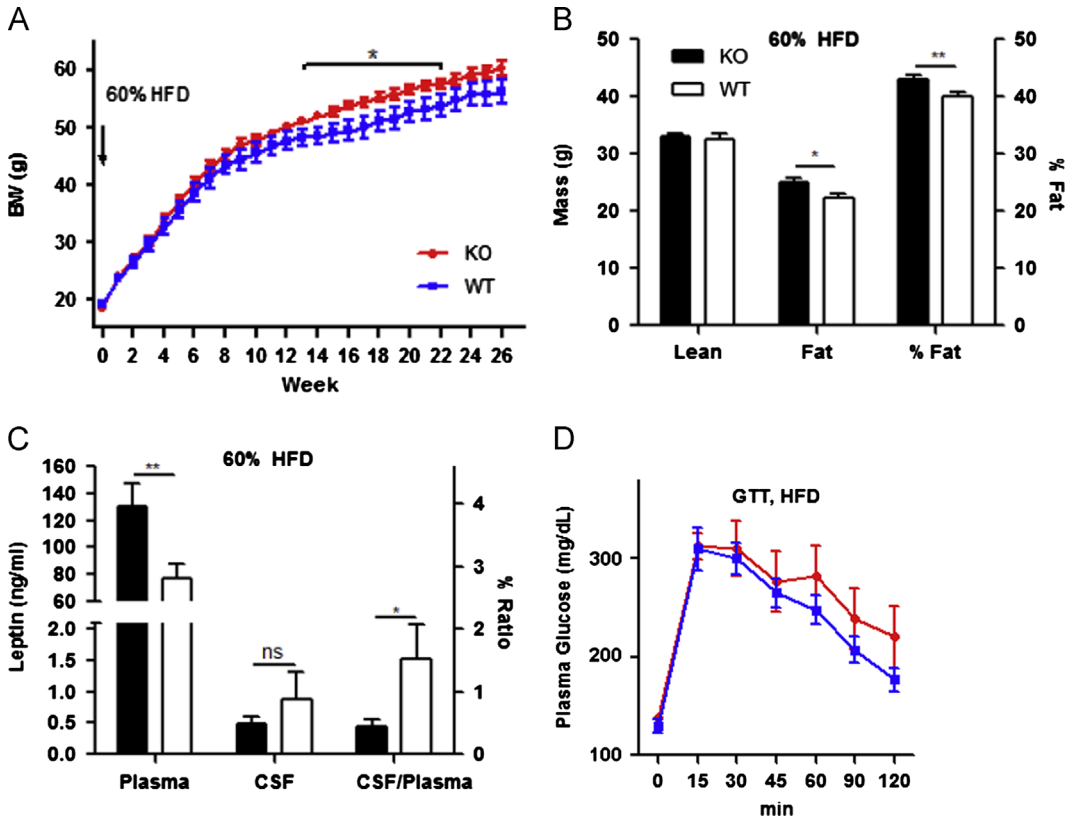

Figure 3: ObRa KO mice show mildly increased susceptibility to diet-induced obesity. ObRa KO ($n=16$) and WT ($n=14$) male littermates were subjected to 60 kcal% fat diet at 6 weeks of age for 26 weeks. Body weight was measured during the course of the treatment. Body composition, GTT, plasma and CSF leptin concentrations were measured by the end of the treatment. (A) ObRa KO mice showed significantly heavier body weight during treatment weeks 13–22 ($p<0.05$ for paired comparison at each time-point; for example, week 16: 53.85 ± 0.75 vs. 49.39 ± 1.83 g). (B) ObRa KO mice showed significantly increased absolute fat mass (25.0 ± 0.7 vs. 22.4 ± 0.6 g, $p<0.05$), percentage of fat mass (43.1 ± 0.6 vs. $40.0\pm0.6\%$, $p<0.01$) and unaltered lean mass (33.0 ± 0.6 vs. 33.5 ± 0.6 g, $p>0.05$). (C) ObRa KO showed significantly more elevated plasma leptin (130.54 ± 16.50 vs. 77.06 ± 11.3 ng/ml, $p<0.01$) and reduced CSF to plasma ratio (0.45 ± 0.10 vs. 1.51 ± 0.58, $p<0.05$), while the difference in CSF leptin was not significant (0.49 ± 0.10 vs. 0.88 ± 0.36 ng/ml, $p>0.05$). (D) ObRa KO and WT mice responded similarly to GTT after HFD ($p>0.05$ for all paired comparisons at each time-point).

5: -4.5 ± 0.5 vs. $-6.7\pm0.7\%$, $p<0.05$; day 9: -5.8 ± 0.6 vs. $-7.9\pm0.8\%$, $p<0.05$). Owing to the fact that KO mice lost less weight during leptin treatment, they in turn gained less weight after pump removal ($p<0.01$ or 0.05 for all time-points after pump-removal; for example, day 1: 2.06 ± 0.42 vs. $4.52\pm0.59\%$; day 9: 9.00 ± 0.46 vs. 12.31 ± 0.95). Food intake showed no significant difference during both phases (Figure 4C, $p>0.05$ at all time-points). These data suggest that ObRa KO mice show a mild leptin resistance with a small but significant decrease in leptin's effects on body weight. This resistance is not as profound as in C57BL/6J db^{ks}/db^{ks} mice with an ObRb-specific mutation or ObR mull mice (db^{3J}/db^{3J} and db^{pas}/db^{pas}), both of which do not show any response to exogenous leptin [12].

3.5. Hypothalamic leptin receptor expression and signaling in ObRa KO mice

We next assayed the levels of ObR mRNA and leptin responsiveness in the hypothalamus of ObRa KO and WT mice. We developed isoform-specific Taqman assays for the c, d and e splice forms of the leptin receptor by aligning the leptin recpetor genomic sequence to the cDNA sequence for each (Figure 5A). We found that ObRc is an un-spliced extension of exon 18, that ObRd is the result of splicing of two short 18-bp exons between exons 19a and 19b, and that ObRe is an un-spliced extension of exon 15. We designed Taqman Assays for ObRc, ObRd and ObRe to cross the boundaries of exons 17 and 18c, 17 and 19d2, and 14 and 15e, respectively.

ObRb is the primary splice variant in the hypothalamus and its levels were unchanged in ObRa KO mice using a transcript-specific Taqman essay (Figure 5B; 0.62 ± 0.03 vs. 0.72 ± 0.05, $p>0.05$). ObRa levels in the hypothalamus were much lower than ObRb in WT animals (0.038 ± 0.002 vs. 0.72 ± 0.05, $p<0.001$), and ObRa transcripts were undetectable in the KO ($2.1e\text{-}05\pm1.7e\text{-}05$ vs. 0.038 ± 0.002, $p<0.001$). The levels of mRNA expression of the other isoforms and total ObR mRNA were also unchanged in the KO, although ObRd mRNA was undetectable (ObRc: 0.56 ± 0.04 vs. 0.48 ± 0.04, $p>0.05$; ObRe: 0.14 ± 0.01 vs. 0.15 ± 0.01, $p>0.05$; total ObR: 0.11 ± 0.01 vs. 0.14 ± 0.01, $p>0.05$). Thus, in the hypothalamus, ObRb is the predominant form and consequently the deletion of the ObRa exon had no discernible impact on ObR mRNA levels in this tissue.

Leptin responsiveness was assayed by analyzing leptin-induced STAT3 phosphorylation in the hypothalamus. Leptin was delivered by acute intra-peritoneal (1 μg/g of body weight, Figure 5C–G) or intra-cerebro-ventricular (20 ng per mouse, Figure 5H–L) injection to male ObRa KO and WT littermates. Leptin-induced STAT3 phosphorylation was assayed by Western blot (whole hypothalamus, Figure 5C, D, H and I) and immunohistochemistry (arcuate nucleus, Figure 5E–G, J–L). As expected, leptin induced significant increases in STAT3 phosphorylation in the whole hypothalamus (Figure 5C and H) and the arcuate nucleus (Figure 5E and J) in both KO and WT. WB blot quantification showed comparable increases in pSTAT3 signal intensity (leptin vs. PBS) between ObRa KO and WT mice (Figure 5C – i.p.: 9.24 ± 0.36 vs.

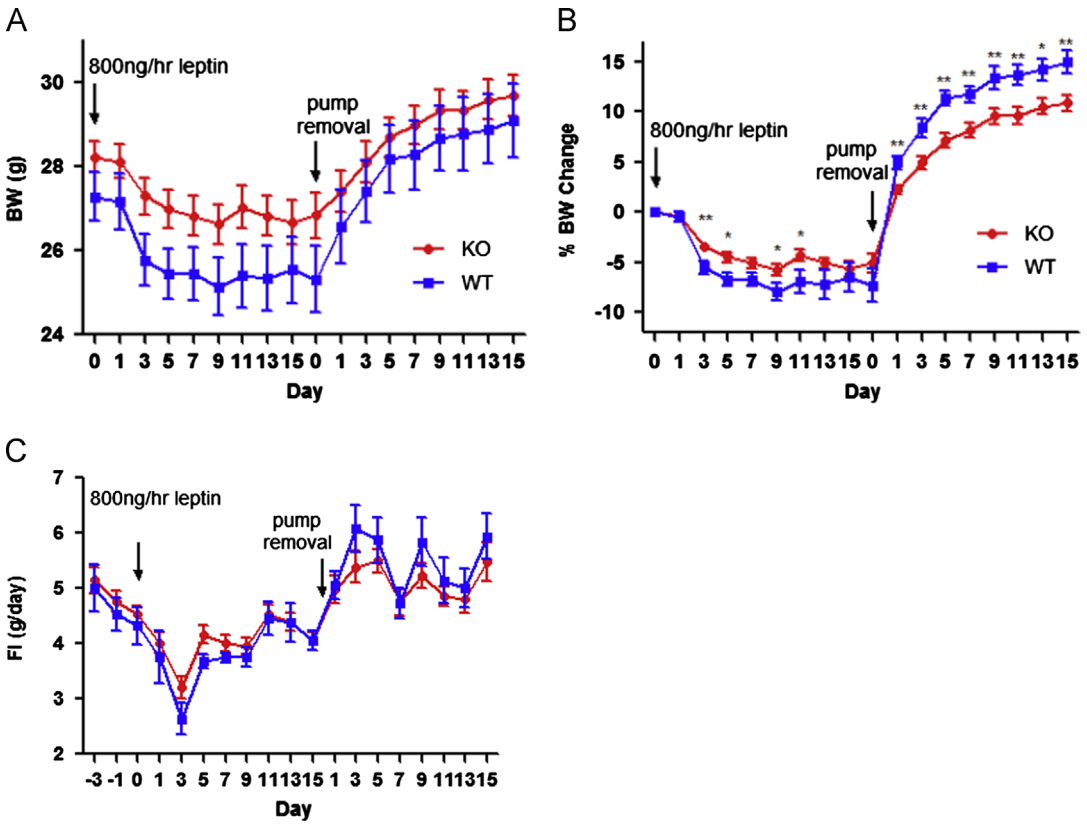

Figure 4: ObRa KO mice show reduced sensitivity to peripherally administered leptin. Sixteen week old ObRa KO ($n=22$) and WT ($n=10$) male littermates were treated with leptin (800 ng/h) delivered by osmotic pumps. Body weight and food intake were measured every other day during and after the infusion. Time of pump insertion and removal are indicated by arrows. (A) Body weight curves of ObRa KO and WT mice showed clear separation starting on day 3 and for the remainder of the treatment, but the difference at each time point did not reach statistical significance (for example, day 3: 27.28 ± 0.43 vs. 25.77 ± 0.61, $p > 0.05$; day 9: 26.61 ± 0.47 vs. 25.13 ± 0.68, $p > 0.05$). (B) ObRa KO mice lost significantly less body weight (expressed as percentage change relative to the BW at day 0) during the phase of leptin infusion (day 3: -3.4 ± 0.4 vs. $-5.5 \pm 0.7\%$, $p < 0.01$; day 5: -4.5 ± 0.5 vs. $-6.7 \pm 0.7\%$, $p < 0.05$; day 9: -5.8 ± 0.6 vs. $-7.9 \pm 0.8\%$, $p < 0.05$) and gained significantly less body weight after the pump removal ($p < 0.01$ or 0.05 during the time course; for example, day 1: 2.06 ± 0.42 vs. $4.52 \pm 0.59\%$; day 9: 9.00 ± 0.46 vs. $12.31 \pm 0.95\%$). (C) ObRa KO and WT mice showed no significant difference in food intake during and after leptin infusion ($p > 0.05$ at all time-points).

9.40 ± 0.73, $p > 0.05$; Figure 5I – i.c.v.: 8.42 ± 0.94 vs. 9.11 ± 1.29, $p > 0.05$). Hippocampi from the same mice were used as a control. As expected, leptin did not lead to significant changes in hippocampal STAT3 phosphorylation in either genotype and there was no significant difference between the two genotypes (i.p.: 1.26 ± 0.18 vs. 1.09 ± 0.16, $p > 0.05$; i.c.v.: 1.66 ± 0.29 vs. 1.12 ± 0.05, $p > 0.05$). In the arcuate nucleus, leptin similarly increased pSTAT3 in both KO and WT, whether quantitated by averaged fluorescence intensity in the region (Figure 5F – i.p. PBS: 301.5 ± 26.6 vs. 241.4 ± 17.9, $p > 0.05$; i.p. Lep: 1138.8 ± 13.5 vs. 1115.5 ± 89.4, $p > 0.05$; Figure 5K – i.c.v. PBS: 756.2 ± 146.2 vs. 921.5 ± 28.1, $p > 0.05$; i.c.v. Lep: 1340.6 ± 44.1 vs. 1479.7 ± 49.2, $p > 0.05$) or number of pSTAT3-positive nuclei (Figure 5G – i.p. PBS: 318 ± 39 vs. 347 ± 70, $p > 0.05$; i.p. Lep: 818 ± 79 vs. 806 ± 62, $p > 0.05$; Figure 5L – i.c.v. PBS: 385 ± 72 vs. 362 ± 102, $p > 0.05$; i.c.v. Lep: 768 ± 42 vs. 752 ± 57, $p > 0.05$).

3.6. Leptin receptor expression in other CNS and peripheral tissues

Finally, we measured the extra-hypothalamic expression of ObRb, ObRc, ObRe and total ObR using the isoform-specific Taqman assays described above (Figure 5A). In contrast to the hypothalamus where the transcript levels of the ObR splice variants were unchanged in the ObRa KO (Figure 5B), there were significant increases in ObRb and ObRc expression in almost all the other tissues from the ObRa KO mice ($p < 0.05$ for all tissues marked with *, Figure 6A and B), while ObRe and total ObR remained unaltered in most tissues (Figure 6D and E). Thus in tissues that express high levels of ObRa, deletion of the ObRa exon results in increases of the ObRc variant which continues past the splice donor of exon 18 as well as the ObRb variant, the exon for which is 3′ to the ObRa and is presumably spliced as a default when the ObRa exon is deleted.

4. DISCUSSION

Leptin signaling is mediated by the leptin receptor, a cytokine family receptor that is spliced into multiple forms. The first splice variants of the leptin receptor that were identified are referred to as ObRa and ObRb. The ObRb form is highly expressed in the hypothalamus and includes a long intra-cytoplasmic region with several motifs capable of activating signal transduction. In contrast, ObRa, the most abundantly expressed form of the leptin receptor in most tissues, expresses a short cytoplasmic region that lacks motifs required for signal transduction. While the requirement of the ObRb form of the leptin receptor for leptin action has been established in numerous studies, little is known about the functional role of ObRa in vivo. To address this, we generated a knockout mouse with a deletion of the ObRa-specific exon. We report that an isoform-specific deletion of ObRa results in a small but significant decrease in leptin responsiveness and a small but significant increase in body weight in mice fed a high fat diet. While these results

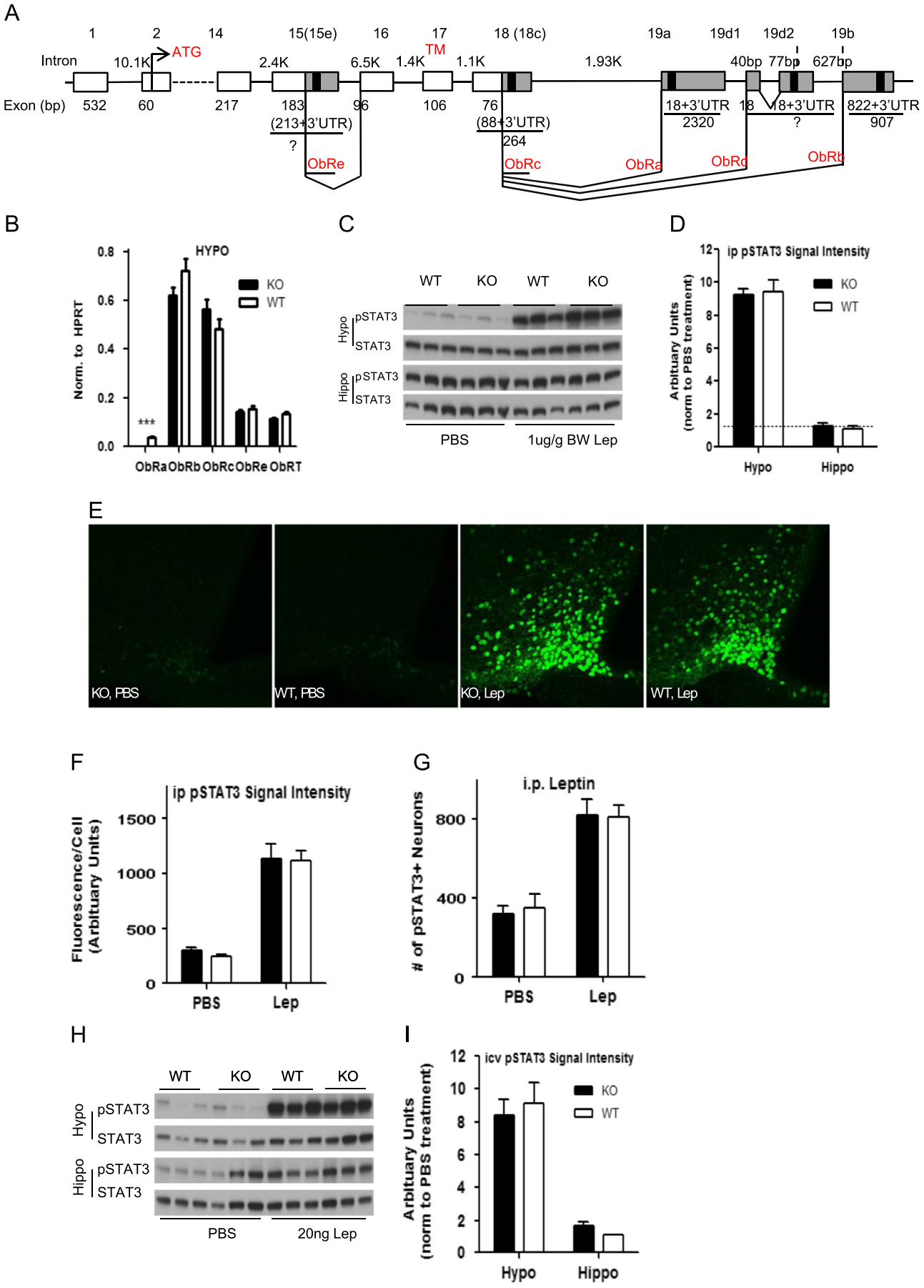

Figure 5: Continued.

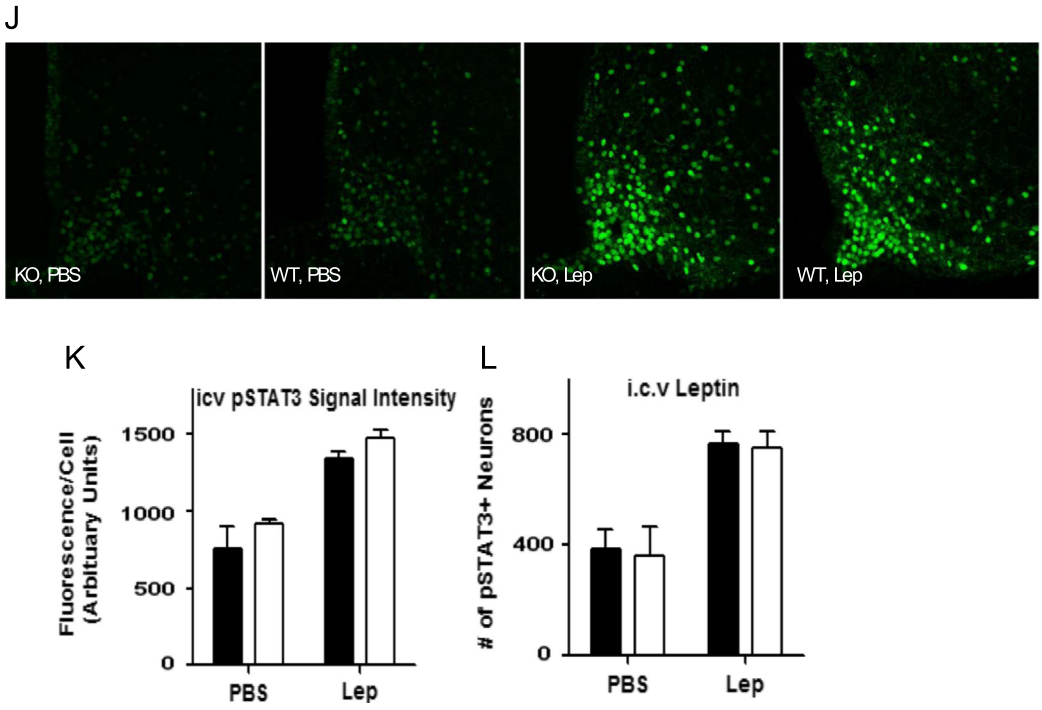

Figure 5: ObRa KO shown unaltered ObRb, ObRc and ObRe expression and STAT3 activation upon leptin administration in the hypothalamus. Hypothalamic ObRb, ObRc, ObRe and total ObR expression were essayed using the same total RNA preparations as used for ObRa in Figure 1B. Another group of ObRa KO and WT male littermates were given 1g/g BW leptin or PBS through i.p. (B–G) or 20 ng leptin in 1 l PBS through i.c.v. injection (G–K). STAT3 activation was essayed by western blot (B, C, G, and H) and immunohistochemistry (D–F, I–K) 45 min after injection using antibodies specific for pSTAT3 (Tyr705) or total STAT3. $n=3$ and 6 for ObRa KO and WT littermates in each treatment group. (A) Diagram illustrating the entire leptin receptor genomic locus with highlights for each of the five alternatively spliced leptin receptor isoforms (Lee Friedman PNAS 1997). Exons are shown by boxes. Alternatively spliced exons are shown by gray boxes, in each of which the presence of the STOP codon is shown by a thick black line. The numbers on the top of the diagram show the numbering of the exons used by UCSC Genome Browser. The sizes of the introns (in Kb) and the exons (in bp) are shown above each intron segment (thin horizontal line) and below each exon, respectively. The position of the ATG (20 bp into exon 2) and the transmembrane domain (encoded by exon 17) are highlighted in red. The sizes of the alternatively spliced exons (15e, 18c, 19a, 19d, and 19b) are underlined. Whenever the actual sequences and sizes of the alternatively spliced exons are known, a single number is labeled underneath. (A) "?" indicates that the size of the corresponding 3'UTR is still inconclusive. 19d1 and 19d2 indicate that after DNA and protein sequence alignment, the terminal exon of ObRd turned out to comprise two 18-bp segments separated by a 77 bp intron. (B) Despite the complete abolishment of ObRa expression in ObRa KO vs. WT mice (2.1e-05 $\pm$ 1.7e-05 vs. 0.038 $\pm$ 0.002, $p<0.001$), ObRb (0.62 $\pm$ 0.03 vs. 0.72 $\pm$ 0.05, $p>0.05$), ObRc (0.56 $\pm$ 0.04 vs. 0.48 $\pm$ 0.04, $p>0.05$), ObRe (0.14 $\pm$ 0.01 vs. 0.15 $\pm$ 0.01, $p>0.05$) and total ObR (0.11 $\pm$ 0.01 vs. 0.14 $\pm$ 0.01, $p>0.05$) showed no significant difference. (C and D) Western blots showing pSTAT3 and total STAT3 signal in hypothalamus and hippocampus from male ObRa KO and WT littermates treated with i.p. leptin and PBS. Leptin-induced pSTAT3 signal (expressed as fold increase relative to pSTAT3 signal intensity under PBS treatment) was comparable between ObRa KO and WT mice (9.24 $\pm$ 0.36 vs. 9.40 $\pm$ 0.73, $p>0.05$). (E) Representative images of pSTAT3 immuno-positive neurons in the arcuate nucleus of ObRa KO and WT mice treated with i.p. leptin or PBS. (F and G) Averaged fluorescence intensity (PBS: 301.5 $\pm$ 26.6 vs. 241.4 $\pm$ 17.9, $p>0.05$; Lep: 1138.8 $\pm$ 13.5 vs. 1115.5 $\pm$ 89.4, $p>0.05$) and cell count (PBS: 318 $\pm$ 39 vs. 347 $\pm$ 70, $p>0.05$; Lep: 818 $\pm$ 79 vs. 806 $\pm$ 62, $p>0.05$) of pSTAT3-positive neurons in the arcuate nucleus. (H and I) Western blots showing pSTAT3 and total STAT3 signal in hypothalamus and hippocampus from male ObRa KO and WT littermates treated with i.c.v. leptin and PBS. Leptin-induced pSTAT3 signal (expressed as fold increase relative to pSTAT3 signal intensity under PBS treatment) showed no difference between ObRa KO and WT mice (8.42 $\pm$ 0.94 vs. 9.11 $\pm$ 1.29, $p>0.05$). (J) Representative images of pSTAT3 immuno-positive neurons in the arcuate nucleus of ObRa KO and WT mice treated with i.c.v. leptin or PBS. (K and L) Averaged fluorescence intensity (PBS: 756.2 $\pm$ 146.2 vs. 921.5 $\pm$ 28.1, $p>0.05$; Lep: 1340.6 $\pm$ 44.1 vs. 1479.7 $\pm$ 49.2, $p>0.05$) and cell count (PBS: 385 $\pm$ 72 vs. 362 $\pm$ 102, $p>0.05$; Lep: 768 $\pm$ 42 vs. 752 $\pm$ 57, $p>0.05$) of pSTAT3-positive neurons in the arcuate nucleus.

are consistent with prior studies indicating that leptin signal transduction is primarily mediated by ObRb, these data do establish a role for ObRa in leptin transport into the CNS and possibly leptin action.

Null mutations of the leptin receptor in db^{3J}/db^{3J} and db Δ (db^{pas}/db^{pas}) mice cause extreme obesity and complete leptin resistance, which are phenotypically identical to that of C57BL/6J db^{ks}/db^{ks} mice which have a mutation only in the ObRb form of the leptin receptor. These data have shown that ObRb is essential for leptin action. This conclusion is consistent with prior reports that point mutations in the C terminus of ObRb also interfere with leptin action [20–22]. While these data establish that ObRb is necessary for leptin action, they do not confirm that it is sufficient in the absence of ObRa and other forms of the leptin receptor. Thus these prior data do not exclude a role for ObRa in leptin trafficking or signal transduction. Indeed, the ObRa form was isolated from choroid plexus suggesting a possible role for the ObRa in leptin transport into or out of the CNS. We find that ObRa KO mice show a small but statistically significant reduction in their (body weight) response to exogenous leptin treatment and a reduced CSF/plasma ratio of leptin in HFD mice, suggesting a possible role for ObRa in leptin transport. We used HFD-treated ObRa KO and WT mice in this experiment, because the levels of CSF leptin in regular chow-fed ObRa KO and WT mice fell below the detection limit of our ELISA (data not shown). HFD treatment significantly increased both plasma and CSF leptin levels in both ObRa KO and WT. Although mean CSF leptin was almost 50% less in ObRa KO vs. WT mice, high variation in WT denied statistically significant. Nonetheless, the ratio of CSF and plasma leptin was significantly reduced in ObRa KO vs. WT mice. These data suggest that ObRa does play a role in leptin transport but that it is not the only means by which leptin accesses the CNS. For instance, leptin-responsive neurons may lie within and project to circumventricular organs (CVO), where the blood brain barrier is permeable and access does not require transport. Thus, the effect of leptin on regions that require blood brain barrier transport may be smaller than the effects of leptin signaling at CVOs. It is also possible that other mechanisms play a role in leptin transport. This latter possibility is consistent with previous studies which showed that obese rodent models deficient in leptin receptor expression or signaling are still capable of leptin uptake into the brain, suggesting that the molecular identity of the leptin transporter is

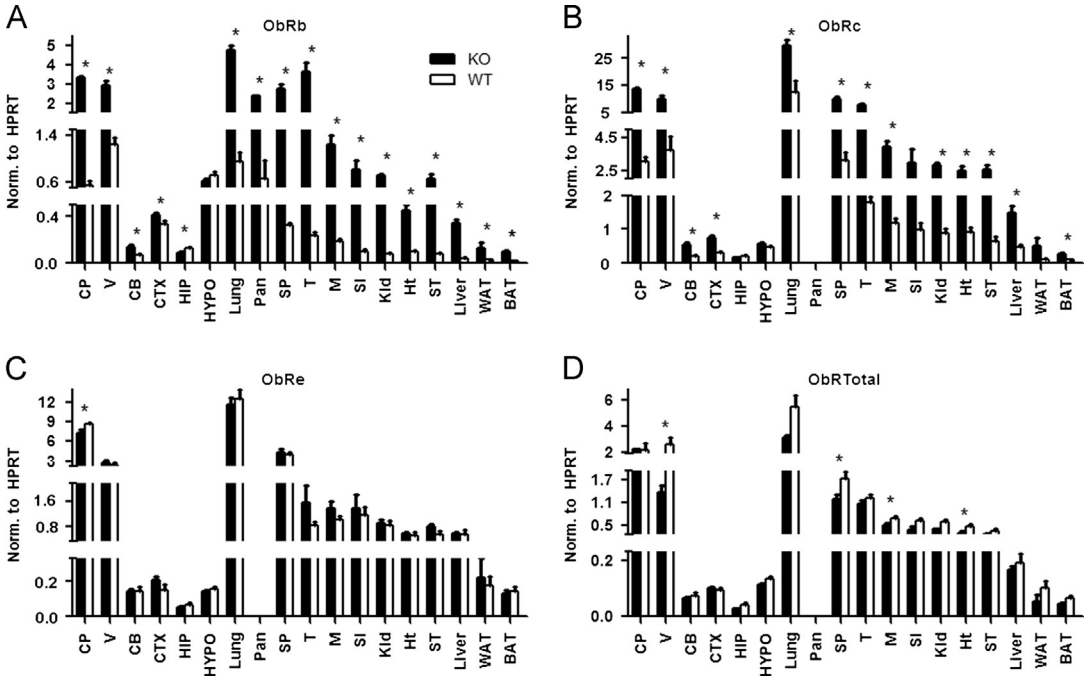

Figure 6: Effect of ObRa KO on leptin receptor expression outside the hypothalamus. ObRb, ObRc, ObRe and total ObR expression were essayed using transcript-specific Taqman essays in the same total RNA preparations as used for ObRa shown in Figure 1B. (A–D) ObRb, ObRc, ObRe and total ObR mRNA levels in all tissues. * indicates that the difference between the KO and WT mice reached statistical significance, regardless of the actual p value < 0.05, 0.01 or 0.001.

still to be identified [8,35,39,40]. Finally, it is also possible that the effect of a deletion of ObRa is to some extent compensated for by the up-regulation of the ObRc splice variant as shown in our ObRa knockout mice. ObRc also expresses a short cytoplasmic region and only differs from ObRa in its three terminal amino acids, and thus could also play a role in leptin transport.

Leptin mediated phospho-Stat3 activation in the hypothalamus is unaltered in the ObRa knockout mice. These data suggest that ObRa is not necessary for leptin's abililty to access hypothalamic neurons and activate Stat3 in this brain region. Neurons in this region are in the vicinity of median eminence (a CVO), which could explain why pStat3 levels are not reduced in the absence of ObRa. This is consistent with the largely unchanged metabolic phenotypes of the ObRa mice. However, ObRa KO mice showed a transient (weeks 13–22) increase in body weight gain vs. WT when fed a high fat diet. By the end of the treatment, ObRa KO showed significantly increased fat accumulation vs. WT albeit similar body weight. This suggests that ObRa may play a role in leptin transport or signal transduction when levels are high (for example resulting from a HFD). This is consistent with our finding that ObRa KO showed a small but significantly reduced body weight loss during system leptin administration, showing that knocking out ObRa in mice leads to a very mild leptin resistance.

Curiously, ObRa KO mice on a chow diet showed significantly reduced fasting glucose and improved GTT, which were not accompanied by changes in plasma insulin. However, ObRa KO and WT mice showed similarly abnormal glucose metabolism on a HFD. It is unclear whether the improved glucose metabolism on a chow diet was secondary to an observed increase in plasma leptin in 14 week old ObRa KO mice. The increased leptin level in these animals suggested that ObRa could have a direct or indirect effect on leptin production or turnover. Similarly, mice with a truncated ObRb (lacking all sequences downstream from the JAK2 binding motif) resemble *db*/*db* mice except that they show a mildly improved glucose homeostasis at young ages [41].

As mentioned above, ObRa KO mice showed significantly up-regulated expression of ObRb and ObRc mRNA in almost all tested tissues and it is possible that the up-regulation of these other forms compensates for the loss of ObRa. Since ObRa, ObRb and ObRc are alternative spliced isoforms that diverge at a common splicing acceptor site at the end of exon 18, the increases in ObRb and ObRc most likely resulted from a compensatory mechanism which regulates the total amount of ObR mRNA expressed in a given tissue (which is likely to be transcriptionally regulated). This is in contrast to the effects on ObR expression in the hypothalamus of ObRa knockout mice where the expression of ObRb and the other ObR isoforms (except ObRa) as well as total ObR is unchanged. These data suggest that a tissue-specific regulation of ObR splicing favors the production of ObRb in the hypothalamus but that other tissues favor the production of ObRa. In the absence of the ObRa exon, however, ObRb and ObRc are instead generated by default splicing events (or no splicing in the case of ObRc). It is unclear whether the significantly up-regulated ObRb and ObRc expression compensates for some of the potential effects of an ObRa KO, and should be noted that total ObR expression remains decreased in micro-vessels, spleen, muscle and heart.

Nonetheless, the broad tissue distribution of ObR mRNA is consistent with previous studies which showed the presence of leptin receptor in various CNS and peripheral organs besides the hypothalamus using leptin binding, an ObRb-cre $\times$ Rosa 26 lox stop lox GFP mouse and immunohistochemistry [8,17,42–54]. It is worth pointing out that although antibodies targeting different epitopes of the leptin receptors have been developed, it remains difficult to distinguish each splice variant from one another, owning to the identical amino acid sequences shared by all splice variants and technical difficulties to generate a strong ObRb C-terminal specific antibody.

5. CONCLUSION

In conclusion, our study provides in-vivo evidence that ObRa plays a small but statistically significant role in mediating leptin action. This effect appears to be independent of leptin's action on the hypothalamus and suggests that ObRa could play a role in mediating leptin's transport, internalization or signal transduction in one or more extra-hypothalamic sites. Meanwhile, these data are consistent with the prior conclusion that most of leptin's actions are mediated by the ObRb form of the receptor. Further studies will be necessary to establish the mechanism by which leptin is transported into the CNS or whether ObRa plays a role in this process and whether the ObRc form can serve some of the same functions as ObRa.

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CONFLICT OF INTEREST

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

APPENDIX A. SUPPLEMENTARY MATERIALS

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.molmet.2013.07.007.

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