



# Mediobasal hypothalamic overexpression of DEPTOR protects against high-fat diet-induced obesity

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## ABSTRACT

**Background/Objective:** The mechanistic target of rapamycin (mTOR) is a serine—threonine kinase that functions into distinct protein complexes (mTORC1 and mTORC2) that regulate energy homeostasis. DEP-domain containing mTOR-interacting protein (DEPTOR) is part of these complexes and is known to dampen mTORC1 function, consequently reducing mTORC1 negative feedbacks and promoting insulin signaling and Akt/PKB activation in several models. Recently, we observed that DEPTOR is expressed in several structures of the brain including the mediobasal hypothalamus (MBH), a region that regulates energy balance. Whether DEPTOR in the MBH plays a functional role in regulating energy balance and hypothalamic insulin signaling has never been tested.

**Methods:** We have generated a novel conditional transgenic mouse model based on the Cre-LoxP system allowing targeted overexpression of DEPTOR. Mice overexpressing DEPTOR in the MBH were subjected to a metabolic phenotyping and MBH insulin signaling was evaluated.

**Results:** We first report that systemic (brain and periphery) overexpression of DEPTOR prevents high-fat diet-induced obesity, improves glucose metabolism and protects against hepatic steatosis. These phenotypes were associated with a reduction in food intake and feed efficiency and an elevation in oxygen consumption. Strikingly, specific overexpression of DEPTOR in the MBH completely recapitulated these phenotypes. DEPTOR overexpression was associated with an increase in hypothalamic insulin signaling, as illustrated by elevated Akt/PKB activation.

**Conclusion:** Altogether, these results support a role for MBH DEPTOR in the regulation of energy balance and metabolism.

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**Keywords** mTOR; DEPTOR; Hypothalamus; Energy balance; Glucose metabolism

## 1. INTRODUCTION

The mechanistic target of rapamycin (mTOR) plays an important role in the hypothalamic regulation of energy balance [1–3]. mTOR is a serine/threonine kinase that nucleates two protein complexes named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [4]. These complexes are part of a well-conserved anabolic pathway that modulates growth and metabolism in response to nutrients and growth factors. They include several proteins, including DEP-domain containing mTOR-interacting protein (DEPTOR), a recently discovered component of mTORC1 and mTORC2 [5]. Studies in several models have shown that DEPTOR promotes insulin sensitivity and protein kinase B (Akt/PKB) activation *in vitro* and *in vivo* [5–9]. Mechanistically, DEPTOR was shown to reduce the negative feedback inhibition of

insulin receptor and improve insulin signaling by dampening mTORC1 activity [5,7,8]. Recently, we mapped the expression of DEPTOR in the rat brain and observed that *Deptor* mRNA was expressed in several structures involved in energy balance regulation, including the mediobasal hypothalamus (MBH) [10]. The MBH includes proopiomelanocortin (POMC) and neuropeptide Y (NPY)/agouti-related protein (AgRP)/aminobutyric acid (GABA)-producing neurons of the arcuate nucleus (ARC), as well as steroidogenic factor-1 (SF-1) neurons of the ventromedial hypothalamus (VMH). In addition to their role in the regulation of energy balance, these neurons have been shown to be involved in systemic glucose homeostasis through their impact on peripheral tissues including the liver and brown adipose tissue [11,12]. Intensive efforts are currently being made to understand how MBH neurons control food intake, energy expenditure and systemic

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metabolism [13,14]. The MBH has additionally emerged as a major center integrating nutrient and hormonal cues to regulate energy balance [13,14].

Whether DEPTOR in the MBH plays a functional role in regulating energy balance has never been reported. In addition, whether DEPTOR promotes hypothalamic Akt/PKB is unknown. In the present study, we report the generation of a new conditional transgenic mouse model based on the Cre-LoxP system allowing targeted overexpression of DEPTOR. Using this model, we found that both systemic and MBH-specific overexpression of DEPTOR protect against high-fat diet-induced obesity and metabolic complications. Given the role of DEPTOR in enhancing peripheral insulin sensitivity and Akt/PKB [5–9], we also delineate how DEPTOR affects the insulin signaling pathway in the MBH and in a neuronal cell line.

## 2. MATERIAL AND METHODS

### 2.1. Animal care

Animal care and handling were performed in accordance with the Canadian Guide for the Care and Use of Laboratory Animals and all experimental procedures received prior approval from the Laval University Animal Care Committee (CPAUL). Male mice were maintained on a 12-h light/dark cycle (lights on 0600–1800), while individually housed in ventilated cages at an ambient temperature of  $23 \pm 1$  °C. They were fed *ad libitum* a chow (Harlan Teklad, 2918) or a high-fat diet (Research Diets, D12492) for 6–8 weeks prior to the experimental procedures. Mice were aged 10–12 weeks at the beginning of the experiments.

### 2.2. Generation of mice

Embryonic stem (ES) cells in which a FRT site was introduced by homologous recombination in the *ColA1* locus were used for the generation of this model. These cells contain a hygromycin resistance gene (HygroR) that lacks a start (ATG) codon. The ES cells were targeted using a vector containing the following elements: 1) a strong mammalian promoter (CAGGS) cloned upstream of a STOP codon surrounded by two LoxP sites, 2) a polyadenylation signal (SV40 pA) placed next to the STOP codon, and 3) the *Deptor* coding sequence cloned downstream of the LoxP-STOP-LoxP cassette. The targeting vector contained a FRT site that is essential for its integration into the *ColA1* locus of the ES cells that also carried a FRT entry site. To allow the entry of the targeting vector into the genomic DNA of the ES cells, these cells were electroporated with an additional vector permitting the expression of the FLP recombinase. In the presence of the FLP protein, the FRT sites recombined and the targeting vector was incorporated in the *ColA1* locus of the ES cells. The presence of a strong PGK promoter upstream of a start codon (ATG) in the targeting vector allowed the expression of the HygroR that is present in the genomic DNA of the ES cells. The expression of this gene is key for the selection of the ES cells that had incorporated the targeting vector. In basal conditions, the presence of a STOP codon upstream of *Deptor* blocks its expression in *Deptor<sup>LoxP-STOP-LoxP</sup>* mice. *Deptor<sup>LoxP-STOP-LoxP</sup>* mice were backcrossed to C57BL/6J mice for at least 10 generations. These mice were crossed with CMVcre mice (The Jackson Laboratory) expressing a cre recombinase in all tissues including the brain.

### 2.3. Stereotaxic AAV2/2 injection

AAV2-Empty and AAV2-Cre were generated by the Molecular Tools Platform of Neurophotonics Centre (Québec, Canada). Surgical procedures were performed under ketamine/xylazine anesthesia as we previously described [15]. Animals were stereotaxically implanted with

a bilateral steel guide cannula (Plastics One) targeting the MBH (5.8 mm depth, 2.8 mm caudal to bregma, 0.4 mm lateral from the sagittal suture). Animals were allowed to recover for 1 week. AAV-Empty or AAV-Cre were bilaterally injected into the MBH ( $2.2 \times 10^{13}$  pfu) via a syringe pump (Harvard Apparatus) at a rate of  $50 \text{ nl min}^{-1}$  for 10 min ( $0.5 \mu\text{l}$  per injection site) based on previously described methodologies [16,17]. Twelve days after injection, mice were fed a chow or a high-fat diet for the duration of the experiments. Efficiency and localization of the bilateral MBH injection was confirmed *post-mortem* using *in situ* hybridization histochemistry.

### 2.4. Food intake and body composition analysis

Body weight and food intake were measured weekly. Body composition was measured by dual-energy X-ray absorptiometry (DEXA) using the PIXIMUS mouse densitometry apparatus (Lunar Corporation, Madison, WI, USA) under isoflurane. Feed efficiency was measured as the ratio of total weight gained (g) divided by energy consumed (kJ).

### 2.5. Metabolic cage experiments

Oxygen consumption ( $\text{VO}_2$ ) and respiratory quotient (RQ) were evaluated over 24 h in an open-circuit system with an  $\text{O}_2$  (S-3A1; Applied Electrochemistry, Naperville, IL) and a  $\text{CO}_2$  analyzer (CD-3A; Applied Electrochemistry). As previously described,  $\text{VO}_2$  data are expressed in  $\text{ml/kg}^{0.75}/\text{min}$  as no differences were observed in lean body mass between the groups [18,19]. The expression of calorimetric data represents a critical and difficult issue that has recently been addressed [19,20], but the consideration of lean body mass is justified, especially when the feed efficiency data suggest an increase in energy expenditure [19,21]. Locomotor activity was measured with the AccuScan Digiscan Activity Monitor (AccuScan Instruments, Columbus, OH) using the VersaMax software (version 1.30; AccuScan Instruments). Physical activity was determined by breaks in photo beams and converted into distance from the horizontal beam (m/24-h period). Mice were placed individually in acrylic chambers for a 72-h adaptation period, and  $\text{VO}_2$ , RQ and movement were measured for 24 h.

### 2.6. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

For the GTT, mice were fasted for 12 h and were injected ip with 1 g/kg of  $\text{D-Glucose}$  (Sigma Aldrich, St Louis, MO). For the ITT, animals were fasted for 6 h and were injected ip with 0.75 U/kg of insulin (Humulin, Lilly, Canada). Blood samples were collected from the tail vein and glucose was measured using a glucometer (OneTouch).

### 2.7. Western blots

Antibodies were obtained from the following sources: antibody to DEPTOR (NBP1-49674) from Novus Biologicals and antibodies to phospho-S473 Akt/PKB (4058), phospho-T308-Akt/PKB (2965), Akt/PKB (4691), S6K1 (9202), phospho-T389 S6K1 (9205), FoxO1 (9462), phospho-T24/T32 FoxO1/3a (9464), S6 (2217), and phospho-Ser240/244 S6 (5364), from Cell Signaling Technology. Secondary antibodies were also purchased from Cell Signaling Technology. Western blots were performed as previously described [10]. Mini-PROTEAN® TGX Stain-Free™ Precast gels (Bio-Rad) were imaged using the Stain-Free application on the ChemiDoc MP (Bio-Rad) imager immediately after the protein separation and prior to western blotting. The ImageLab software version 4.1 (Bio-Rad) was used to select and determine the background-subtracted density of the bands, which were corrected using a total protein quantitation approach as described previously [22]. For MBH experiments, mice were fasted for 12 h followed by a 1 h refeeding.

### 2.8. Gene expression

Total mRNA was isolated from brown adipose tissue (BAT) and liver using PureZOL™ RNA isolation reagent and the Aurum™ Total RNA Fatty and Fibrous Tissue kit (Bio-Rad). Total mRNA was isolated from cells using E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek). The RNA concentrations were estimated from absorbance at 260 nm. cDNA synthesis was performed using the iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad) as described [23]. mRNA extraction and cDNA synthesis were performed following the manufacturer's instructions. cDNA was diluted in DNase-free water (1:25) before the quantification by real-time PCR. mRNA transcript levels were measured in duplicate samples using a CFX96 touch™ real-time PCR (Bio-Rad, Mississauga, ON, Canada). Chemical detection of the PCR products was achieved with SYBR Green (Bio-Rad, 172–5271). At the end of each run, melt curve analyses were performed, and representative samples of each experimental group were run on agarose gels to ensure the specificity of the amplification, as previously described [23]. Fold differences in target mRNA expression were measured using the  $2^{-\Delta\Delta}$ -cycle threshold method by comparison with the housekeeping gene acidic ribosomal phosphoprotein (*Arbp*) and expressed as fold change vs. controls.

### 2.9. Brain *in situ* hybridization histochemistry

The protocol for *in situ* hybridization has been previously described [10]. A mouse-specific *Deptor* cDNA probe was generated from a 266-bp fragment of the 5'-region of DEPTOR sub-cloned into a pGEM-T plasmid (Stratagene, La Jolla, CA), and linearized with SphI and NcoI (Pharmacia Bio-tech, Baie-d'Urfée, QC, Canada) for antisense and sense probes, respectively.

### 2.10. Cell culture

Immortalized hypothalamic GT1-7 cells were kindly provided by Dr. Pamela Mellon (UCSD, San Diego, California, USA) [24]. Overexpression of DEPTOR was induced using a CMV-driven construct as described previously [5]. Cells were starved from serum for 12 h prior to being exposed to serum for 2 h or indicated doses of insulin for 20 min. Each experiment was conducted at least three times.

### 2.11. Statistical analysis

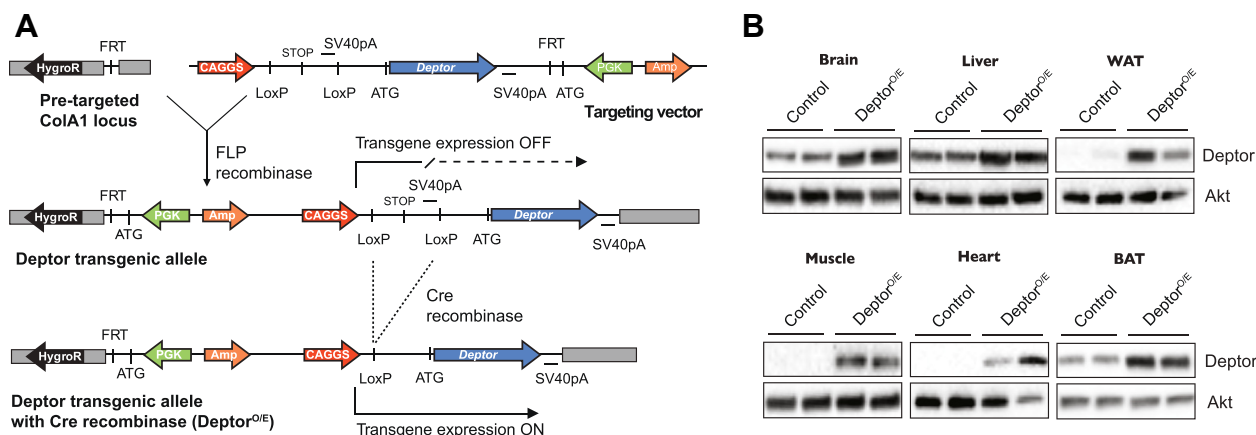
Results are expressed as means  $\pm$  SEM. Statistical analysis of differences was performed using Graph Pad Prism Software version 6.0 for Mac (San Diego, CA, USA). The two-tailed Student's *t*-test for non-paired values was used for two group comparisons. Two or more groups were compared using ANOVA followed by Bonferroni post hoc test. A *p*-value  $<0.05$  was considered statistically significant.

## 3. RESULTS

### 3.1. Generation of a conditional *Deptor* transgenic mouse model

We have recently reported the generation of a doxycycline-inducible DEPTOR transgenic mouse [7]. This model has proven extremely useful to study the role of DEPTOR *in vivo*. However, our model did not allow any overexpression of DEPTOR in the brain [7]. In fact, the doxycycline-inducible DEPTOR mouse model we used was developed by inserting in the *Col1A1* locus a single *Deptor* transgene flanked by a tetracycline operator (TetO), as reported before [25,46]. Although this approach was shown to be efficient to achieve high overexpression in several tissues, many groups, including our own, observed that this model could not be used to express the transgene in the brain [7,25,46]. The exact reason why doxycycline does not allow the expression of the transgene in the brain of this model is unknown. Nevertheless, to circumvent this limitation, we have generated a new transgenic mouse for conditional overexpression of DEPTOR. A schematic view of the targeting strategy is presented in Figure 1A. Based on a previously described method [25], we have inserted in the *Col1A1* locus a single-copy of a transgene composed of a CAGGS promoter, a *LoxP*-stop-*LoxP* cassette and the *Deptor* coding sequence. In the presence of a *Cre* recombinase, the stop codon surrounded by *LoxP* site is eliminated, which allows overexpression of the *Deptor* transgene (Figure 1A).

To study the physiological impact of systemic and constitutive DEPTOR overexpression *in vivo*, mice carrying the transgenic allele were crossed with mice expressing the *Cre* recombinase under the control of a human cytomegalovirus (CMV) minimal promoter (*CMV-Cre*). In this strain, the *Cre* gene is expressed early during embryogenesis and



**Figure 1: Generation of a *Deptor* transgenic mouse.** (A) The transgenic *Deptor* allele containing a strong mammalian promoter (CAGGS) cloned upstream of a STOP codon surrounded by two *LoxP* sites and the *Deptor* coding sequence cloned downstream of the *LoxP*-STOP-*LoxP* cassette was integrated into the *Col1A1* locus of embryonic stem (ES) cells. In basal conditions, the presence of a STOP codon upstream of *Deptor* blocks its expression in *Deptor<sup>LoxP-STOP-LoxP</sup>* mice. However, when these mice are crossed with a mouse expressing a *cre* recombinase, the *LoxP* sites recombine, the STOP codon is eliminated and *Deptor* expression from the transgene is allowed. (B) Western blot showing the expression of DEPTOR in tissues of *Deptor<sup>LoxP-STOP-LoxP</sup>* mice crossed with *CMVcre* mice expressing the *cre*-recombinase in all tissues (*Deptor<sup>OE</sup>*). Akt/PKB was used as a loading control.

recombination of *LoxP* sites occurs in all tissues [26]. As depicted in Figure 1B, DEPTOR overexpressor (*Deptor*<sup>O/E</sup>) mice showed elevated DEPTOR protein levels in all tissues, including the brain. These results confirm the validity of our targeting strategy and establish this model as a valuable alternative to study the role of DEPTOR in this organ.

### 3.2. Whole-body DEPTOR overexpression increases locomotor activity and improves glucose homeostasis in lean mice fed a standard diet

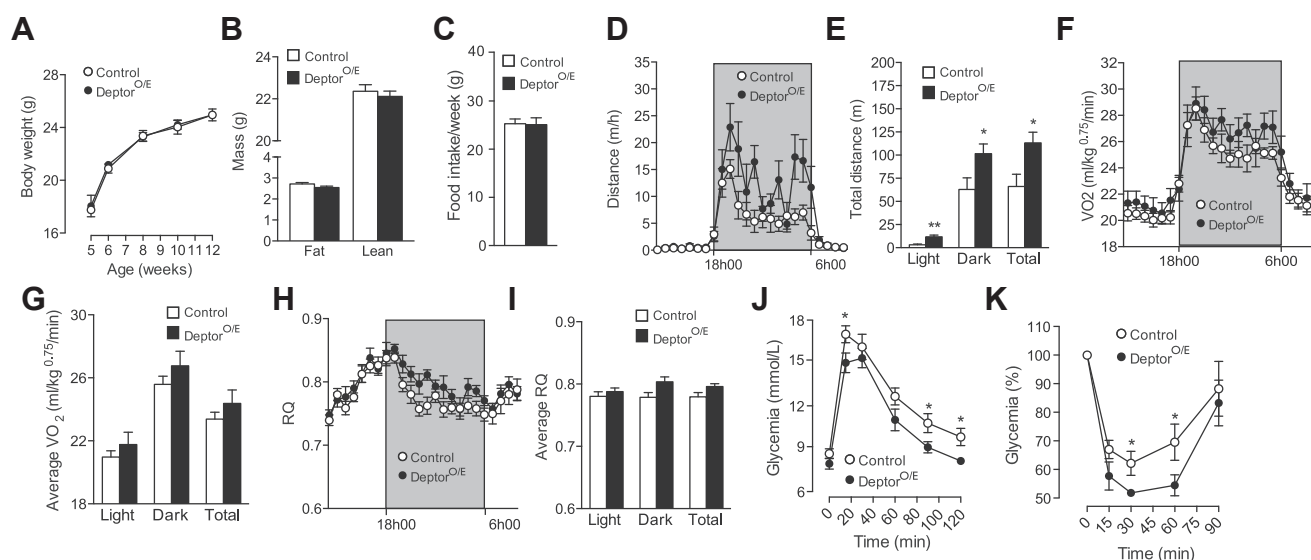
*Deptor*<sup>O/E</sup> mice were born at the expected Mendelian ratio and did not show apparent physiological difference when fed a laboratory chow (Figure 2A). We did not measure any change in body composition and food intake between the groups (Figure 2B,C). Interestingly, we observed during routine husbandry care that *Deptor*<sup>O/E</sup> mice appeared to be more active than their control littermates. To test the accuracy of this observation, we placed mice in metabolic chambers where locomotor activity, as well as energy expenditure, were recorded. This experiment showed that DEPTOR overexpression significantly increased activity, an effect that was particularly obvious during the dark phase (Figure 2D,E). Despite elevated locomotor activity, we did not detect significant variation in oxygen consumption and the respiratory quotient (RQ) between *Deptor*<sup>O/E</sup> and control mice (Figure 2F–I). This suggests that the elevation in physical activity observed in *Deptor*<sup>O/E</sup> mice might not have been sufficient to affect global energy balance and body weight over the course of the experiments.

DEPTOR is an important regulator of the mTOR pathway, a signaling node playing key roles in energy metabolism [7]. Based on previous studies indicating that hypothalamic mTOR signaling directly affects glucose tolerance and insulin sensitivity [27,28], we tested whether DEPTOR overexpression affects glucose homeostasis by performing glucose and insulin tolerance tests (GTT and ITT, respectively) on *Deptor*<sup>O/E</sup> and control mice. Interestingly, the clearance of glucose was improved during the GTT in *Deptor*<sup>O/E</sup> mice (Figure 2J). We also observed that insulin was more efficient in reducing blood glucose levels in these animals (Figure 2K). Together, these results show that

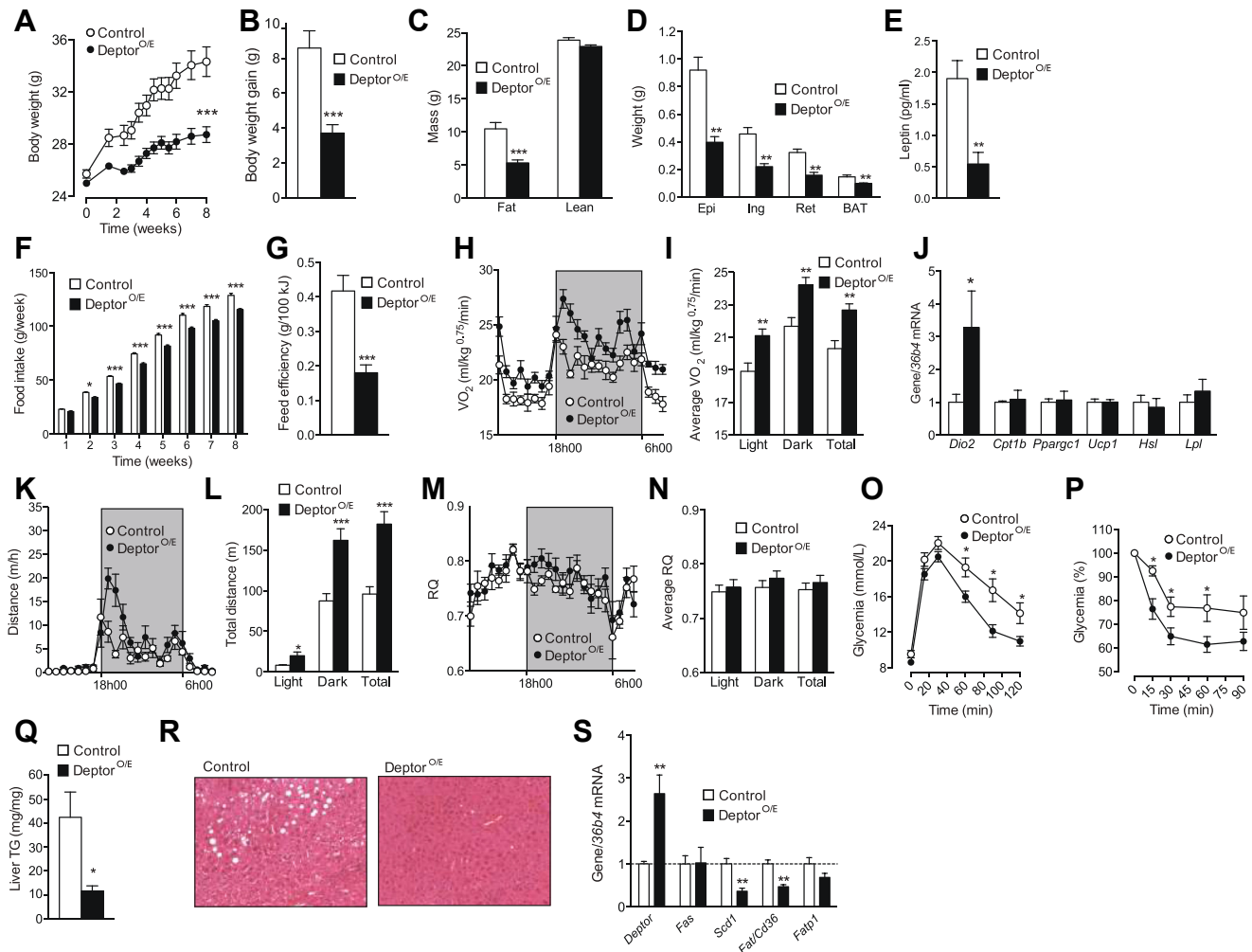
DEPTOR overexpression improves glucose homeostasis, even in lean and healthy young mice fed a standard laboratory chow. Importantly, improvement in glucose metabolism and elevation in locomotor activity are phenotypes that were not previously observed in mice overexpressing DEPTOR only in peripheral tissues [7], thus suggesting that DEPTOR expression in the brain could be required for these effects.

### 3.3. *Deptor*<sup>O/E</sup> mice are protected against high-fat diet-induced obesity and metabolic alterations

To characterize the metabolic consequences associated with systemic and constitutive DEPTOR overexpression, *Deptor*<sup>O/E</sup> mice were challenged with a high-fat diet. *Deptor*<sup>O/E</sup> mice gained significantly less weight than their littermates when fed a high-fat diet (Figure 3A,B). A significant reduction in fat, but not lean mass, was observed in *Deptor*<sup>O/E</sup> mice (Figure 3C,D). Consistent with a reduction in fat mass, plasma leptin levels were reduced in *Deptor*<sup>O/E</sup> mice (Figure 3E). This phenotype was associated with a reduction in food intake and feed efficiency and an elevation in oxygen consumption when considering the important difference of body weight between the groups (Figure 3F–I). Supporting this observation, the expression of *type 2 iodothyronine deiodinase (Dio2)*, which is associated with adaptive thermogenesis in brown adipose tissue [29], was significantly increased in *Deptor*<sup>O/E</sup> mice (Figure 3J). As observed in chow-fed animals, locomotor activity was higher in *Deptor*<sup>O/E</sup> mice fed a high-fat diet (Figure 3K and L) but no effect on the RQ was observed in *Deptor*<sup>O/E</sup> and control mice (Figure 3M,N). Furthermore, DEPTOR overexpression prevented high-fat diet-induced glucose intolerance and insulin resistance, as evidenced by the improved GTT and ITT profiles (Figure 3O,P). We also observed that *Deptor*<sup>O/E</sup> mice had lower liver triglyceride content (Figure 3Q,R). This effect was associated with a reduction in the expression of *fatty acid translocase (Fat/Cd36)* and *stearoyl-CoA desaturase 1 (Scd1)*, two genes regulating lipid uptake and synthesis in hepatocytes (Figure 3S). Altogether, these results indicate that, while preventing obesity by reducing food intake and increasing energy expenditure, DEPTOR overexpression protects mice



**Figure 2: Phenotype of *Deptor*<sup>O/E</sup> mice.** (A) Body weight of mice fed with a chow diet. (B) Body composition of mice fed with a chow diet aged 11–12 weeks. (C) Average weekly food intake of mice fed with a chow diet aged 11–12 weeks. (D) Physical activity expressed in meter per hour and (E) total traveled distance of mice fed with a chow diet aged 9–10 weeks. (F) Oxygen consumption and (G) average oxygen consumption of mice fed with a chow diet aged 10–11 weeks. (H) Respiratory quotient (RQ) and (I) average RQ of mice fed with a chow diet aged 9–10 weeks. (J) Glucose tolerance test (GTT) and (K) insulin tolerance test (ITT) of mice fed with a chow diet aged 9–10 weeks. The data are expressed as the mean  $\pm$  SEM for  $n = 10$ – $12$  (for A–C and J–K) and  $n = 8$  (for D–I). \*\* $p < 0.01$  and \* $p < 0.05$  versus control. Control mice represent the littermates.



**Figure 3: *Deptor*<sup>O/E</sup> mice are protected against high-fat diet-induced obesity and metabolic alterations.** (A) Body weight and (B) body weight gain of mice fed with a high-fat (HF) diet for 8 weeks. (C) Body composition of mice fed with a HF diet for 8 weeks. (D) Weight of the different fat depots of mice fed with a HF diet for 8 weeks. (E) Fasting plasma leptin levels of mice fed with a HF diet for 8 weeks. (F) Cumulative food intake and (G) feed efficiency of mice fed with a HF diet for 8 weeks. (H) Oxygen consumption and (I) average oxygen consumption of mice fed with a HF diet for 8 weeks. (J) Expression of thermogenic genes in BAT of mice fed a HF diet for 8 weeks. (K) Distance traveled expressed in meter per hour and (L) total traveled distance of mice fed with a HF diet for 8 weeks. (M) Respiratory quotient (RQ) and (N) average RQ of mice fed with a HF diet for 8 weeks. (O) Glucose tolerance test (GTT) and (P) insulin tolerance test (ITT) of mice fed with a HF diet for 6 weeks. (Q) Liver triglyceride (TG) content of mice fed a HF diet for 8 weeks. (R) H&E stained sections from livers of mice fed a HF diet for 8 weeks. (S) Liver expression of genes involved in lipid metabolism of mice fed a HF diet for 8 weeks. The data are expressed as the mean  $\pm$  SEM for  $n = 10-12$  (for A–D),  $n = 5-6$  (for E, R–T) and  $n = 8-12$  (for F–Q). \*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$  versus control. Control mice represent the littermates.

against the development of metabolic complications including glucose intolerance, insulin resistance and hepatosteatosis.

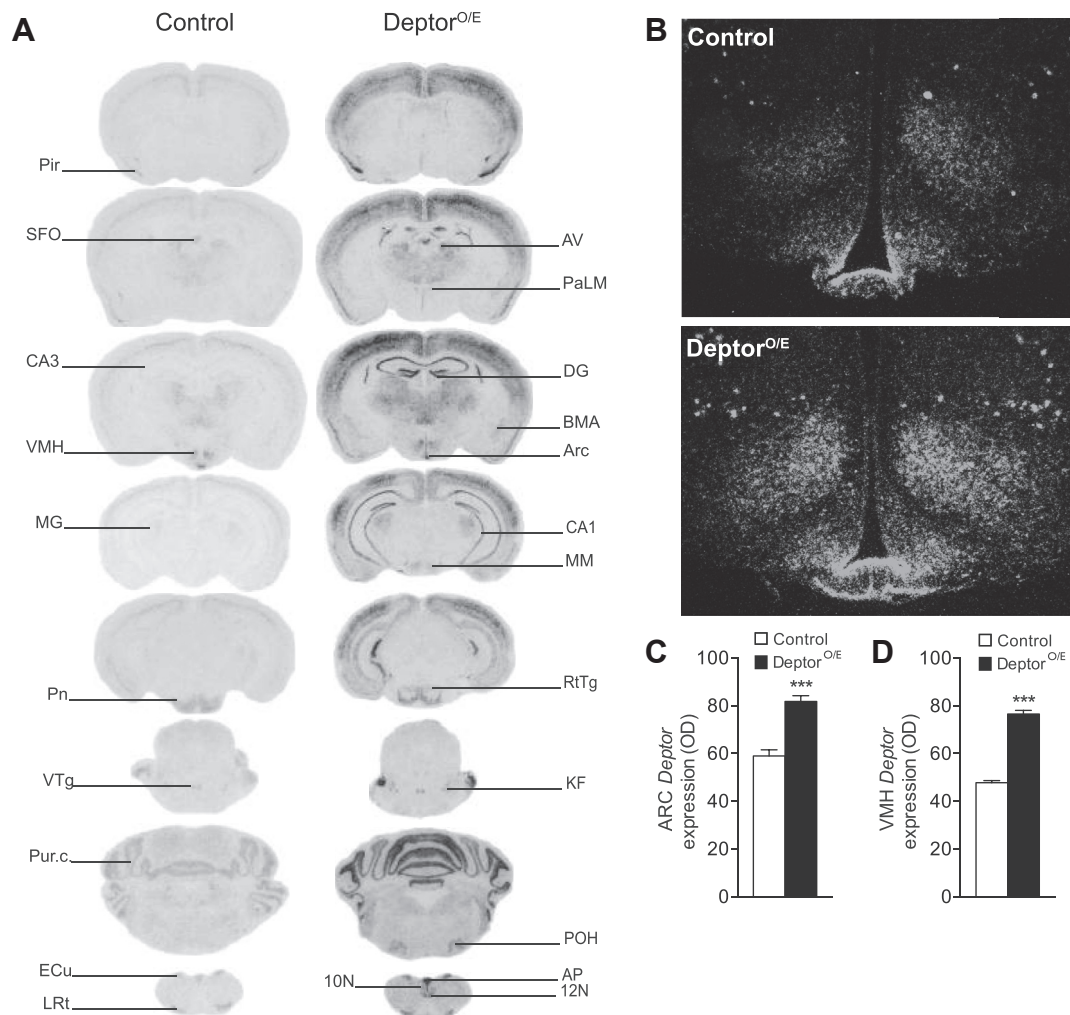
### 3.4. *Deptor* is expressed in key regions of the brain regulating energy balance

The hyperactivity, resistance to obesity and the improvement in glucose homeostasis observed in *Deptor*<sup>O/E</sup> mice are phenotypes that were not observed when DEPTOR was overexpressed only in peripheral tissues [7]. This prompted us to question whether these phenotypes could be linked to the overexpression of DEPTOR in the brain. We have recently described the distribution profile of *Deptor* in the rat brain [10]. In rats, *Deptor* is widely expressed from the forebrain to the hindbrain, with high expression in the MBH (ARC and VMH), the hippocampus [dentate gyrus (DG) and CA3 field], and the circumventricular organs [subfornical organ (SFO), median eminence (ME) and area postrema (AP)] [10]. As shown in Figure 4A, the brain

distribution of *Deptor* in mouse followed the same pattern. Interestingly, *Deptor*<sup>O/E</sup> mice showed a significant increase in *Deptor* expression in most of these regions. Precisely, *Deptor*<sup>O/E</sup> mice exhibited a 28% and 38% increase in *Deptor* expression in the ARC (Figure 4B,C) and the VMH (Figure 4B,D), which are key regions of the MBH known to control food intake, energy expenditure and systemic glucose metabolism [13]. These changes were associated with a similar and physiological elevation in DEPTOR protein levels in the MBH (Figure 1B).

### 3.5. MBH-specific DEPTOR overexpression protects mice against obesity

In order to determine the contribution of MBH DEPTOR to the phenotype observed in whole-body *Deptor*<sup>O/E</sup> mice, mice harboring the *LoxP-stop-LoxP* cassette and the *Deptor* coding sequence were injected bilaterally in the MBH with adeno-associated virus (AAV) encoding the



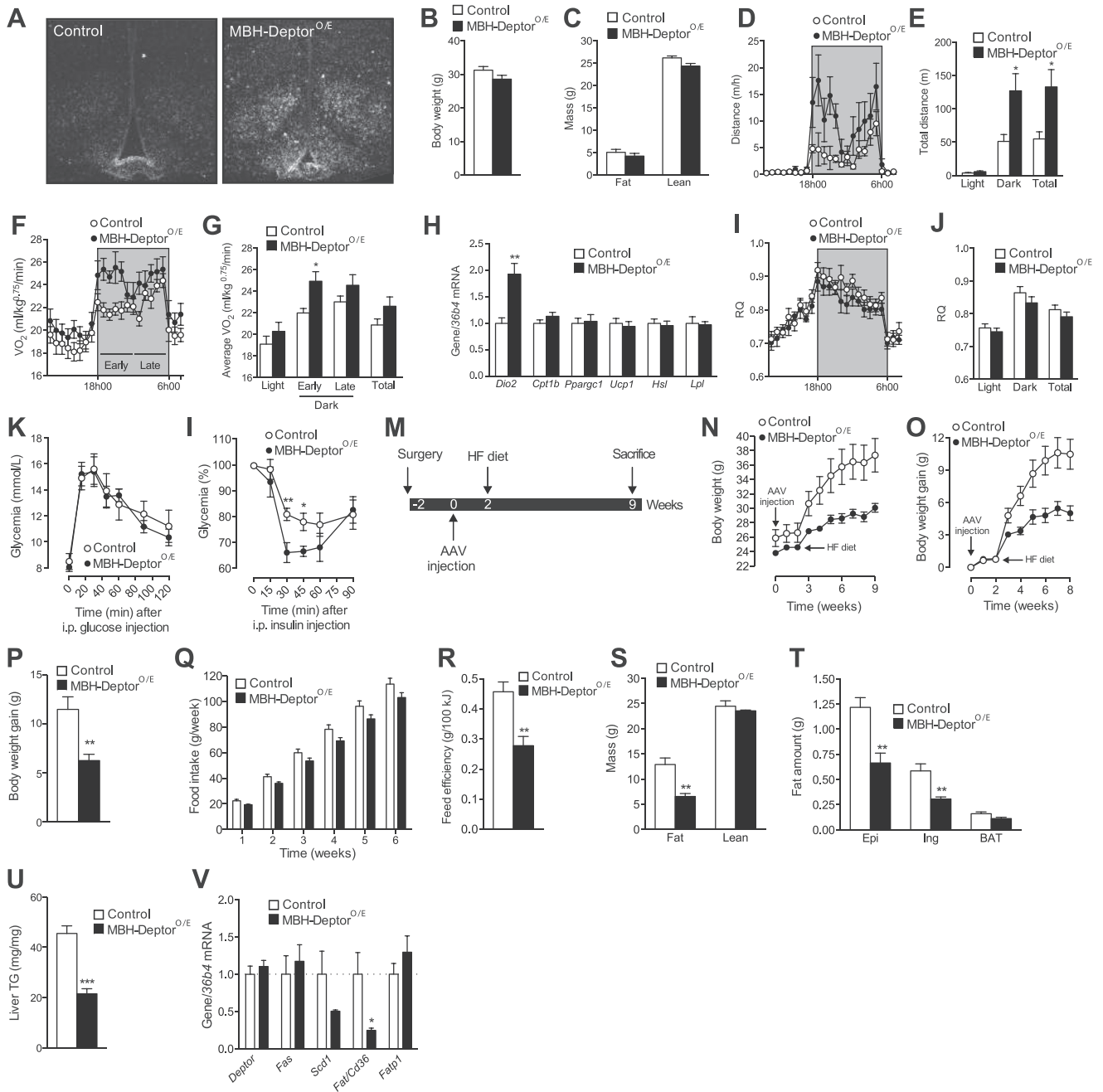
**Figure 4: *Deptor* is expressed in key regions of the brain regulating energy balance.** (A) Rostrocaudal distribution of *Deptor* in *Deptor*<sup>O/E</sup> and control mice. The coronal brain sections appearing in the distribution were photographed from a film autoradiogram. The photographs were mounted on cardboard and the brain regions identified and labeled. The identity of the labeled regions was ascertained under microscope using the emulsion-dipped sections that were used for the photographs. (B) Darkfield photomicrographs of coronal brain sections comparing *Deptor* mRNA expression in the mediobasal hypothalamus of *Deptor*<sup>O/E</sup> and control mice. (C) *Deptor* mRNA expression in the ARC and (D) VMH of *Deptor*<sup>O/E</sup> and control mice aged 20–22 weeks. The data represent means  $\pm$  SEM. \*\*\* $P < 0.001$  as assessed by Student's *t*-test. 10N, dorsal motor nucleus of the vagus nerve; 12N, hypoglossal nucleus; AP, area postrema; Arc, arcuate nucleus of the hypothalamus; AV, anteroventral thalamic nucleus; BMA, basomedial amygdaloid nucleus; CA1/3, field CA1/3 of the hippocampus; DG, dentate gyrus; ECu, external cuneate nucleus; KF, Killiker-Fuse nucleus; LRt, lateral reticular nucleus; MG, medial geniculate nucleus; MM, medial mammillary nucleus; PaLM, paraventricular lateral magnocellular; Pir, piriform cortex; Pn, pontine nuclei; POH, periolivary horn; Pur.c, Purkinje cell; RTg, reticulotegmental nucleus of the pons; SFO, subfornical organ; VMH, ventromedial hypothalamus; VTg, ventral tegmental nucleus.

Cre recombinase to produce MBH-specific *Deptor*<sup>O/E</sup> mice (MBH-*Deptor*<sup>O/E</sup>). Control mice were injected with empty viruses. *In situ* hybridization showed that this approach efficiently promotes *Deptor* expression in the ARC and the VMH (Figure 5A). In this model, DEPTOR overexpression was restricted to the MBH and not present in other regions of the brain or peripheral tissues.

In line with what was observed in chow-fed *Deptor*<sup>O/E</sup> mice, we did not measure any change in body weight and body composition in laboratory chow-fed MBH-*Deptor*<sup>O/E</sup> mice (Figure 5B,C). However, locomotor activity was significantly higher in the latter (Figure 5D,E). Interestingly, chow-fed MBH-*Deptor*<sup>O/E</sup> mice exhibited increased oxygen consumption, mainly at the beginning of the dark phase, further supporting a role for MBH DEPTOR in the control of energy expenditure (Figure 5F,G). As observed in *Deptor*<sup>O/E</sup> mice, *Dio2* expression was significantly increased in the interscapular brown adipose tissue of MBH-*Deptor*<sup>O/E</sup> mice (Figure 5H). Again, no effect on the RQ was

measured between the groups (Figure 5I,J). Even though MBH-specific DEPTOR overexpression did not seem to affect glucose tolerance as assessed by GTT (Figure 5K), it significantly improved insulin sensitivity in chow-fed animals (Figure 5L). These results indicate that MBH DEPTOR plays a prominent role in the phenotype observed in whole-body *Deptor*<sup>O/E</sup> mice.

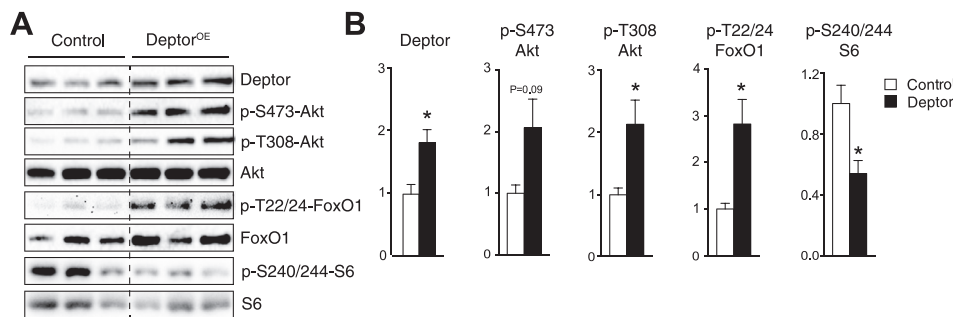
To test whether MBH DEPTOR expression could prevent diet-induced obesity, as observed in whole-body *Deptor*<sup>O/E</sup> mice, MBH-*Deptor*<sup>O/E</sup> mice were challenged with a high-fat diet (Figure 5M). Strikingly, MBH-*Deptor*<sup>O/E</sup> mice gained less weight when exposed to a high-fat diet (Figure 5O–N). These mice did not eat significantly less ( $p = 0.097$ ) but exhibited a reduction in feed efficiency, which suggests an increase in energy expenditure (Figure 5Q,R). It is noteworthy that a significant reduction in fat, but not lean mass, was observed in MBH-*Deptor*<sup>O/E</sup> mice, together with a reduction in liver triglyceride content (Figure 5S–U). Similar to what was observed in whole-body *Deptor*<sup>O/E</sup>,



the reduction in hepatosteatosis was associated with lower expression in *Fat/Cd36* and *Scd1* mRNA (Figure 5V). Altogether, these results indicate that MBH DEPTOR expression is sufficient to recapitulate most of the phenotypes observed in *Deptor*<sup>O/E</sup> mice, including resistance to high-fat diet-induced obesity, increased feed efficiency, improvement in insulin sensitivity, and resistance to hepatosteatosis.

### 3.6. DEPTOR overexpression promotes hypothalamic Akt/PKB phosphorylation

DEPTOR has been previously shown to improve insulin signaling in cancer cells and preadipocytes by relieving mTORC1/S6K1-mediated feedback inhibition of the insulin receptor [5,7]. In these studies, it was shown that DEPTOR overexpression increases Akt/PKB



**Figure 6: DEPTOR overexpression activates Akt/PKB in the hypothalamus.** (A) Protein expression in MBH of chow-fed *Deptor*<sup>O/E</sup> mice after 12 h of fasting followed by 1 h of refeeding. (B) Quantification of protein expression in the MBH of *Deptor*<sup>O/E</sup> mice. The data are expressed as the mean ± SEM for n = 3–5 per condition. \*p < 0.05 versus control. Control mice represent the littermates.

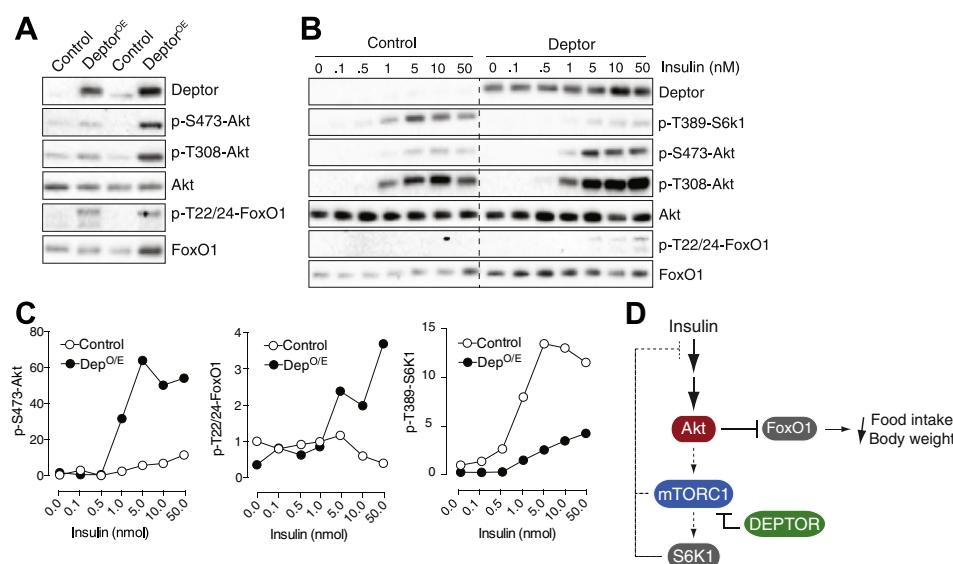
phosphorylation, a key readout activated by insulin. Whether DEPTOR modulates Akt/PKB activation centrally has never been investigated. Proteins were extracted from the MBH of control and *Deptor*<sup>O/E</sup> mice and western blot analyses were performed. We observed that DEPTOR overexpression increased MBH Akt/PKB phosphorylation on both hydrophobic S473 and catalytic T308, which are targets of mTORC2 and PDK1, respectively (Figure 6). The phosphorylation of Forkhead box O1 (FoxO1), a transcription factor directly targeted by Akt/PKB, was also significantly increased in the MBH of *Deptor*<sup>O/E</sup> mice (Figure 6). Interestingly, we observed a reduction in the phosphorylation of S6, a classical readout of mTORC1 activity. Importantly, these results were also reproduced *in vitro* using murine hypothalamic GT1-7 neurons that stably overexpressed DEPTOR (Figure 7A–C). Using this standard model [16], we observed that DEPTOR overexpression increased Akt/PKB phosphorylation, an effect associated with the reduction in S6K1 phosphorylation. Although GT1-7 neuronal cells are derived from gonadotropin-releasing hormone (GnRH) cells and not from neurons of the MBH, the experiments performed using these cells allowed us to define a general molecular link between DEPTOR overexpression and insulin signaling. Altogether, these results suggest that DEPTOR

overexpression rewires insulin signaling, not only in the periphery [7] but also in the MBH and in neuronal GT1-7 cells (Figure 7D).

#### 4. DISCUSSION

Improving the understanding of the network that regulates energy balance appears to be a prerequisite to understand the pathophysiology of obesity and related disorders [30]. Here, we provide evidence that DEPTOR plays a role in energy balance regulation and systemic metabolism. Observations made using transgenic mouse models indicate that MBH DEPTOR overexpression protects mice against obesity and the development of obesity-associated metabolic complications.

Given the brain distribution of *Deptor* mRNA and protein recently reported in rats [10], it seemed probable that DEPTOR might play a significant role in energy homeostasis. Here we show that *Deptor* is also widely expressed in the mouse brain. Interestingly, the brain distribution of *Deptor* in mice is extremely similar to that recently described in rats, indicating a high degree of conservation between these species [10]. Of note, we found that *Deptor* is highly expressed in



**Figure 7: DEPTOR overexpression increases Akt/PKB in murine hypothalamic GT1-7 cells.** (A) Protein expression of components of the insulin signaling pathway in control GT1-7 cells and GT1-7 cells stably overexpressing DEPTOR (B–C) Protein expression of components of the insulin signaling pathway in control GT1-7 cells and GT1-7 cells overexpressing DEPTOR following various low-doses of insulin. (D) Graphical representations of insulin sensitivity and maximal responsiveness following various low-doses of insulin in control and GT1-7 cells overexpressing Deptor. The data are expressed as the mean ± SEM for n = 3–6 per condition. \*\*p < 0.001 and \*p < 0.05 versus control. Control mice represent the littermates (for A).



the MBH, a key region of the brain that integrates signals from circulating nutrients and hormones to regulate food intake, energy expenditure and glucose metabolism [31]. These results clearly suggest a possible role for the protein in the regulation of metabolic processes regulated by the MBH. The MBH hosts important neuronal populations involved in energy balance regulation, including (but not restricted to) POMC and AgRP/NPY neurons in the ARC and SF1 neurons in the VMH [31]. These neurons are recognized as critical in integrating the metabolic signals from leptin and insulin in order to control both food intake and energy efficiency [30,31]. The protection against high-fat diet-induced obesity and the improvement in metabolism observed in mice overexpressing DEPTOR specifically in the MBH highlights the importance of DEPTOR in regulating the function of some of these neurons. Future studies using transgenic mice expressing a cre recombinase under the control of specific promoters such as AgRP, POMC or SF-1 will be required to identify the neurons affected by DEPTOR. Upcoming experiments aimed at defining in details the impact of DEPTOR on specific neuronal populations represents an interesting challenge that will certainly help defining the role of this protein in regulating systemic energy homeostasis. The present results nonetheless offer sound evidence for a metabolic/homeostatic role of MBH DEPTOR in energy balance regulation.

Here, we observed that moderate overexpression of DEPTOR in the MBH deeply affects feed efficiency, which indicates that DEPTOR increases systemic energy expenditure. We also observed that overexpression of DEPTOR increases locomotor activity. The increase in locomotor activity was not symptomatic of any brain illness as the animals ate and behaved normally. It is important to point out that the elevation in activity is unlikely to explain the phenotype of resistance to obesity since laboratory chow-fed animals of similar body weight also exhibit an increase in movement without a reduction in feed efficiency. These observations suggest that overexpressing DEPTOR in the MBH could positively affect other energy expenditure components such as brown adipose tissue thermogenesis. As such, we observed that oxygen consumption was increased during the early dark phase, suggesting an increase in energy expenditure. In addition, expression of *Dio2* was increased in both systemic and MBH models, suggesting that the thermogenic capacity of brown adipose tissue was higher [29]. As others [29,32] and we [23,33] have demonstrated, *Dio2* represents one of the most readily activated genes in conditions enhancing BAT thermogenesis.

Brain insulin signaling likely plays a role in regulating energy balance and glucose homeostasis [34,35]. Neuron-specific insulin receptor knockout mice develop obesity and insulin resistance [36]. Moreover, deletion of the p110 $\alpha$  subunit of PI3K in neurons also results in increased body weight due to decreased energy expenditure [14,37]. Supporting these observations, insulin suppresses feeding and improves glucose homeostasis by stimulating specific neurons in the hypothalamus [35,38]. In agreement with previous studies [5–8], we observed an increase in MBH Akt/PKB phosphorylation in response to DEPTOR overexpression, indicative of an increase in hypothalamic insulin signaling. Interestingly, this effect was associated with resistance to diet-induced obesity and an improvement in systemic glucose metabolism, mimicking an effect associated with elevated central insulin action. Interestingly, it was recently demonstrated that reduction in hypothalamic Akt/PKB caused by the loss of *Rictor*, an essential component of mTORC2, impairs glucose homeostasis in mice and causes obesity [39]. These results support the possibility that MBH DEPTOR could promote leanness and improve glucose metabolism by promoting Akt/PKB activation. Interestingly, the increase in MBH Akt/PKB activity following DEPTOR overexpression was also observed in

immortalized hypothalamic GT1-7 cells, reinforcing the idea that DEPTOR overexpression rewires insulin signaling, not only in the periphery [7] but also in the MBH and in neuronal cells. Although one could consider these cells to be different from the well-characterized MBH neurons, they represent a useful tool to better investigate molecular events downstream of metabolic receptors [16,38,40].

Several studies have highlighted the importance of mTORC1 in the hypothalamic regulation of energy balance [1–3]. Within neurons of the MBH, mTORC1 activity is induced by food intake and repressed by fasting [1]. There is also evidence indicating that key hormones known to affect energy balance, such as leptin, insulin and ghrelin, modulate mTORC1 signaling in the MBH [1,38,41]. However, recent studies failed to identify mTORC1 as a regulator of energy balance [28,42]. These studies further revealed that mTORC1 in specific MBH neurons affects peripheral glucose metabolism but is dispensable for the control of feeding behavior and energy metabolism. Although DEPTOR was initially introduced as an mTOR inhibitor [5], its role in regulating mTOR signaling is extremely complex [7,43,44]. In fact, DEPTOR does not impair mTORC1 activity like rapamycin does, but only dampens its activation, which is sufficient to relieve the feedback inhibition of PI3K and activate Akt/PKB activity. Even though DEPTOR physically interacts with mTORC2 and efficiently reduces Akt/PKB phosphorylation in kinase assays performed in tubes [5], a reduction in Akt/PKB phosphorylation has not been observed in response to DEPTOR overexpression in tissues, *in vivo*. The ability of DEPTOR to relieve the negative feedback loops from mTORC1 to PI3K appears sufficient to promote Akt/PKB phosphorylation by PDK1 on T308, an event that facilitates its phosphorylation on S473 by mTORC2. Supporting this model, several studies performed in mice showed that one important consequence linked to DEPTOR expression in peripheral tissues is the elevation in Akt/PKB phosphorylation on both T308 and S473 [7,45]. Our results indicate that DEPTOR similarly activates Akt/PKB in the brain.

We have previously developed a doxycycline-inducible DEPTOR transgenic mouse to study the role of DEPTOR *in vivo* [7]. Using this model, we reported that mice overexpressing DEPTOR only in peripheral tissues but not in the brain become obese when fed a high-fat diet. We showed that DEPTOR cell-autonomously promotes adipogenesis by activating peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) in adipocytes, an effect associated with Akt/PKB activation [7]. The inability of doxycycline to induce the expression of DEPTOR centrally in this mouse model has, however, prevented its use to study the role of DEPTOR in the brain [7,25]. Here, we found that systemic (brain and periphery) overexpression of DEPTOR does not cause obesity but rather protects mice against this condition. Strikingly, MBH-specific DEPTOR overexpression also protects mice against high-fat-induced obesity, thus indicating that hypothalamic DEPTOR plays a central role in this effect. Although these observations do not rule out the importance of DEPTOR in the cell-autonomous regulation of adipogenesis, they indicate that hypothalamic DEPTOR, through its impact on energy balance regulation, plays a dominant role in controlling adiposity. The fact that peripheral overexpression of DEPTOR promotes fat accumulation is not surprising since increased insulin signaling and Akt/PKB activation is well known to promote anabolic processes such as lipogenesis and adipogenesis [47–51]. On the other hand, elevation in insulin signaling in the brain favors a catabolic phenotype characterized by a reduction in food intake and an increase in energy expenditure [35], just as described here. Altogether, the studies support the notion that Akt/PKB activation promotes anabolism in the periphery and catabolism in the brain and that DEPTOR could play an important role in regulating these functions.

In summary, the present study reports that systemic overexpression of DEPTOR prevents high-fat diet-induced obesity, improves glucose metabolism and protects against the development of metabolic disturbances. These phenotypes were associated with a reduction in feed efficiency. Strikingly, specific overexpression of DEPTOR in the MBH completely recapitulated these phenotypes, thus supporting a key role for MBH DEPTOR in the regulation of energy balance and metabolism.

### CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

### AUTHOR CONTRIBUTIONS

Conceptualization, A.C., D.R. and M.L.; Methodology, A.C., D.R. and M.L.; Investigation, A.C., S.M.L., D.L., P.-G.B. R.V., and C.R.; Resources, D.M.S., D.R. and M.L.; Writing — Original Draft, A.C. and M.L., Writing — Review & Editing, A.C. D.R. and M.L.; Supervision, D.R. and M.L., Funding Acquisition, D.R. and M.L.

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