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Hypothalamic tanycytes are an ERK-gated conduit for leptin into the brain

Eglantine Balland[1], **Julie Dam**[#2], **Fanny Langlet**[#1], **Emilie Caron**[#1], **Sophie Steculorum**[#1], **Andrea Messina**[1], **S Rasika**[1], **Anthony Falluel-Morel**[3], **Youssef Anouar**[3], **Bénédicte Dehouck**[1], **Eric Trinquet**[4], **Ralf Jockers**[2], **Sebastien G Bouret**[1,5], and **Vincent Prévot**[1,*]

[1] Inserm UMR837, Jean-Pierre Aubert Research Centre, UDSL, IMPRT, 59000 Lille, France

[2] Inserm U1016, CNRS UMR 8104, Institut Cochin, Univ. Paris Descartes, 75000 Paris, France

[3] Inserm U982, University of Rouen, 76821 Mont-Saint-Aignan, France

[4] Cisbio Bioassays, Parc Technologique Marcel Boiteux, BP84175, 30200 Codolet, France

[5] Neuroscience Program, The Saban Research Institute, Children's Hospital Los Angeles, University of Southern California, Los Angeles, CA 90027, USA

[#] These authors contributed equally to this work.

SUMMARY

Leptin secreted by adipocytes acts on the brain to reduce food intake by regulating neuronal activity in the mediobasal hypothalamus (MBH). Obesity is associated with resistance to high circulating leptin levels. Here, we demonstrate that peripherally administered leptin activates its receptor (LepR) in median eminence tanycytes followed by MBH neurons, a process requiring tanycytic ERK signaling and the passage of leptin through the cerebrospinal fluid. In mice lacking the signal-transducing LepRb isoform or with diet-induced obesity, leptin taken up by tanycytes accumulates in the median eminence and fails to reach the MBH. Triggering ERK signaling in tanycytes with EGF reestablishes leptin transport, elicits MBH neuron activation and energy expenditure in obese animals, and accelerates the restoration of leptin sensitivity upon the return to a normal-fat diet. ERK-dependent leptin transport by tanycytes could thus play a critical role in the pathophysiology of leptin resistance, and holds therapeutic potential for treating obesity.

INTRODUCTION

Leptin, a 16kDa peptide hormone mainly produced by adipocytes, is present in serum at levels directly proportional to the amount of adipose tissue, and controls food intake by activating LepR signaling in the brain (Ahima and Flier, 2000; de Luca et al., 2005). In leptin-deficient humans and mice, leptin administration effectively reduces hyperphagia and obesity (Campfield et al., 1995; Farooqi et al., 1999; Halaas et al., 1995; Pelleymounter et al., 1995). Paradoxically, most cases of obesity display high circulating leptin levels that fail

[*] Correspondence: vincent.prevot@inserm.fr.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and Supplemental Experimental Procedures.

to reduce appetite or increase energy expenditure (Frederich et al., 1995). The mechanisms underlying this leptin resistance are unclear, but potentially include its defective transport across the blood-brain barrier to the cerebrospinal fluid (CSF) (Caro et al., 1996; Schwartz et al., 1996) or to its sites of action within the CNS (Van Heek et al., 1997), and failure of the LepR signaling cascade (El-Haschimi et al., 2000; Enriori et al., 2007; Munzberg and Myers, 2005).

Exogenous leptin given peripherally, e.g. intraperitoneally (i.p.), first activates select neurons in the hypothalamic arcuate nucleus (ARH) (Faouzi et al., 2007), a major leptin-sensing site (Coppari et al., 2005; Cowley et al., 2001) , and after a 1-2h lag, triggers LepR signaling in more dorsal hypothalamic sites that control energy homeostasis (Faouzi et al., 2007). Intriguingly, leptin given intracerebroventricularly (i.c.v.) activates all leptin-responsive hypothalamic neurons within minutes (Faouzi et al., 2007), suggesting that the passage of leptin from the periphery to the CSF could act as an important physiological checkpoint in the intrahypothalamic propagation of leptin signaling.

We have recently shown that tanycytes, specialized hypothalamic glia located in a circumventricular organ adjoining the ARH - the median eminence (ME) - and extending from the ependymal surface of the 3rd ventricle to a plexus of permeable fenestrated vessels at the pial surface of the brain, form a barrier between the blood and CSF (Langlet et al., 2013; Mullier et al., 2010). Although structural changes to this barrier modulate the access of blood-borne metabolic signals to ventromedial ARH neurons and, consequently, adaptive responses to acute nutritional challenges (Langlet et al., 2013; Schaeffer et al., 2013), the process by which these factors gain access to hypothalamic circuits that regulate energy balance under normal conditions is largely unknown.

Here, we explored the idea that the ME could serve as the route for leptin entry into the hypothalamus, and that tanycytes man this checkpoint. In addition, we investigated whether leptin transport is altered in mice with diet-induced obesity (DIO) and by what molecular mechanisms. Lastly, we asked whether normal transport could be restored by pharmacological means, and if this rescue would improve leptin sensitivity in DIO mice.

RESULTS

The ME is the first hypothalamic site reached by peripheral leptin

Leptinergic signaling involves various pathways, including STAT3, ERK and PI3K→Akt (Munzberg and Myers, 2005). We studied LepR signaling activation in the ME of adult wild-type (WT) C57Bl/6 mice by immunohistochemistry for phosphorylated STAT3 (pSTAT3) at various times after peripheral leptin administration (3mg/kg). Strikingly, 5 min after i.p. injection, pSTAT3 immunoreactivity (IR) was detected in both tanycytic processes, which contact the capillary plexus at the pial surface of the brain (Langlet et al., 2013; Mullier et al., 2010), and their nuclei, whereas pSTAT3-IR in neurons was infrequent and weak (Figure 1A). By 15 min after injection, while some tanycytes were still labeled, pSTAT3-IR in neurons dramatically increased; at longer intervals, pSTAT3-IR occurred almost exclusively in neurons (Figures 1A and 1B). Thus, circulating leptin appears to sequentially activate LepR in ME tanycytes followed by neurons.

Next, we studied the role of the ME in hypothalamic leptin uptake by western blotting of the ME and of the MBH containing the ARH, microdissected from mice 5, 15 and 45 min after leptin injection i.p. (Figure S1A and S1B). Exogenous leptin was detected only in the ME at 5 min (Figure S1C) but progressively invaded the MBH at 15 and 45 min (Figure 1C). Concurrently, STAT3 was activated in the ME alone at 15 min and in both the ME and MBH at 45 min; pSTAT3 was not detectable at earlier time points in either compartment

(Figure 1C; Figure S1C and S1D), suggesting that the ME is the first hypothalamic site accessed by peripheral leptin.

To determine whether DIO (El-Haschimi et al., 2000; Enriori et al., 2007) altered leptin uptake, we redid these experiments in mice with leptin resistance following a 9-week high-fat diet (Figure S2A and S2B). DIO profoundly disrupted leptin access to the MBH but not the ME, although leptin failed to activate STAT3 in the ME of these mice (Figure 1C). Instead, as previously shown (Enriori et al., 2011; Martin et al., 2006), there was some constitutive STAT3 activity in the MBH (Figure 1C), including in numerous ARH neurons, and to a lesser extent in ME tanycytes, in vehicle- and leptin-treated DIO mice (Figure 1B; Figure S2C). However, neither cell type displayed any further increase in pSTAT3-IR following leptin treatment, suggesting that leptin resistance involves defective LepR signaling in both hypothalamic tanycytes and neurons.

Interestingly, mice lacking the signaling-competent LepRb isoform (*db/db* mice) phenocopied DIO mice, with blood-borne exogenous leptin remaining blocked in the ME in both lean juveniles and obese adults (Figure S1E; Figure 1C). Next, we injected standard-chow-fed WT mice with a mutated recombinant leptin antagonist (LAN) with no biological activity but unmodified LepR-binding properties (Niv-Spector et al., 2005), i.p. While LAN was as readily detected by western blotting as bioactive leptin (Figure S1G), it did not accumulate in the ME or MBH (Figure 1D), confirming that LepR function is required for peripheral leptin to access the ME and MBH.

Blood-borne leptin is taken up by ME tanycytes

To determine the role of tanycytes in hypothalamic leptin uptake *in vivo*, we intravenously administered fluorescently-labeled bioactive leptin (16kDa) (Vauthier et al., 2013) to standard-chow-fed WT mice 5 min before sacrifice. At this short interval, fluorescent leptin was seen exclusively in ME tanycytes in the brain parenchyma, and virtually all ME tanycytes were labeled (Figure 1E). While this labeling occurred mainly in tanycytic cell bodies along the floor of the 3rd ventricle, in a few cases, a tanycytic process stretching to the pial surface was also labeled, indicating that blood-borne leptin is likely taken up by tanycytic end-feet and transported towards the cell body in contact with the CSF (Figure 1E). In contrast, fluorescently-labeled LAN was never seen in tanycytic cell bodies or processes, indicating that LepR activation is required for this uptake.

Passage of blood-borne leptin to the CSF is required for STAT3 activation in MBH neurons

We next examined the putative contribution of the passage of systemic leptin into the CSF to STAT3 activation in MBH neurons *in vivo*, by administering neutralizing antibodies to leptin (20μg/2μl/animal) i.c.v. 10 min before injecting leptin (3mg/kg i.p., 45min). Importantly, neutralizing antibodies did not diffuse into the hypothalamic tissue, but were sometimes found to bind lightly to ME tanycytes (Figure S2D). This neutralization of leptin in the CSF, while it might not have been extensive or exhaustive, significantly hampered the leptin-induced increase in neuronal p-STAT3-IR in the ARH, ventromedial (VMH) and dorsomedial (DMH) nuclei of the hypothalamus (Figure 1F), indicating that the passage of leptin from the blood to the CSF is implicated in the leptin-mediated activation of hypothalamic neurons.

Tanycytic LepR is required for leptin uptake

We further explored the role of tanycytic LepR signaling in the passage of peripheral leptin to the hypothalamus using primary cultures of rat tanycytes, which we have previously extensively characterized (Prevot et al., 2003). RT-PCR and sequence analysis revealed that cultured tanycytes expressed mRNAs for LepRa, b, c and e (Figure 2A). Importantly, after

15 min of leptin treatment, tanycytes displayed STAT3, Akt and ERK phosphorylation (Figure 2B), demonstrating functional LepRb signaling. In addition, cultured tanycytes internalized fluorescent bioactive leptin through clathrin-coated vesicles, known to exist in tanycytes *in vivo* (Peruzzo et al., 2004), as shown by immunocytochemistry (Figure 2C) and western blotting of immunoprecipitated clathrin-coated vesicles (Figure 2D). However, this leptin internalization was blocked neither by selective pharmacological antagonists to the various LepRb-related signaling pathways (U0126 to inhibit MEKK1, the upstream activator of ERK, LY294002 to inhibit PI3K and WP1066 to inhibit STAT3) (Figure 2D; Figure S3A), nor by AG490, an inhibitor of JAK2 autophosphorylation, the well-studied "first" event in the LepR signaling cascade (data not shown). In conjunction with the lack of LAN uptake (Figure 2D), these results strongly indicate that leptin internalization involves an as-yet unexplored LepR-dependant but JAK2-independent mechanism.

Tanycytic release of captured leptin requires ERK signaling

Next, we assessed the fate of fluorescent bioactive leptin, once internalized, using videomicroscopy. Cultured tanycytes are highly polarized, like their *in vivo* counterparts, with cell bodies attached to the coverslip and extending ~20μm-long processes into the medium. Fluorescent leptin was taken up by tanycytic end-feet and gradually transported towards the cell body, where it accumulated (Figure 2E), confirming our *in vivo* observations of labeled processes touching the pial surface and demonstrating that, contrary to previous assumptions (Peruzzo et al., 2004), the default direction for clathrin-mediated transport in tanycytes is basal→apical. Intriguingly, the intensity of the fluorescent signal decreased over time to reach extinction (Figure 2E), suggesting that captured leptin was eventually released. Accordingly, when tanycytes were loaded with leptin (1μg/ml, 15 min), washed with PBS, and leptin release monitored 5 and 15 min later, leptin levels in the medium gradually increased (Figure 2F; Figure S3B), a process involved intracellular vesicular trafficking, as shown by its reversible suppression by colchicine (Figure S3B). While LY294002 and WP1066 had no effect, U0126 led to leptin accumulation in tanycytes (Figure 2F), indicating that its release requires LepRb-ERK signaling.

ERK signaling activation in tanycytes rescues leptin transport and function in the MBH of obese mice

We next verified whether defective leptin translocation from the ME to the MBH of obese mice could be rectified by activating the ERK pathway and thus reinstating leptin release using epidermal growth factor (EGF) to activate ERK because of the abundant expression of erbB1 in tanycytes (Prevot et al., 2003). EGF treatment (1mg/kg, 15 min) markedly activated ERK in ME tanycytes (Figure 3A). Strikingly, leptin treatment followed by EGF acutely restored peripheral leptin uptake by the MBH of *db/db* and DIO mice (Figure 3B) as well as STAT3 activation in hypothalamic neurons of DIO mice (Figure 3C). In contrast, replacing DIO mice on a normal diet, previously shown to normalize body weight and restore leptin sensitivity over time (Enriori et al., 2007), only restored leptin transport into the hypothalamus after several weeks (Figures 4A-C). Daily EGF treatment (i.p., 1mg/kg) in DIO mice during the last week of their high-fat diet, which significantly increased energy expenditure and locomotor activity (Figure 3D-F), also accelerated weight loss (with significant effects visible within 1 week of EGF treatment vs. 3 weeks with dietary changes alone) and restoration of leptin sensitivity upon their return to a normal diet (Figures 4A-C). These data raise the exciting possibility that ERK activation could rescue leptin signaling and function in obese mice with diet-induced leptin resistance (Coppari et al., 2005; Mesaros et al., 2008).

DISCUSSION

Leptin transport into the brain appears to be a limiting step in the modulation of its central effects, and participates in leptin resistance both in humans and rodents (Caro et al., 1996; El-Haschimi et al., 2000; Schwartz et al., 1996; Van Heek et al., 1997). Our findings suggest, intriguingly, that blood-borne leptin enters the brain through the hypothalamic ME, and that tanycytes, which capture leptin from the bloodstream, act as a checkpoint along this route. Indeed our finding that leptin-neutralizing antibodies infused into the 3rd ventricle impede hypothalamic neuron activation indicates that tanycyte-mediated leptin transport between the periphery and the CSF is necessary for its downstream effects. Technological advances that allow the assessment subtle and rapid physiological variations in leptin levels in tiny volumes of CSF will no doubt help confirm or refine these observations. Once past this tanycytic checkpoint and in the CSF, the polarity and barrier properties of ARH tanycytes, which differ from those of ME tanycytes (Mullier et al., 2010), could allow leptin and possibly other blood-borne signals to freely enter the ARH and, subsequently, other hypothalamic regions, by paracellular diffusion. Interestingly, tanycytic cell bodies lining the 3rd ventricle wall at the level of the ME are non-ciliated (Mullier et al., 2010), unlike their more dorsal counterparts (Mullier et al., 2010), potentially creating a quiescent zone that limits signal dispersal through the CSF and creates a favorable diffusion gradient for their entry into the adjacent ARH. However, beyond the immediate vicinity of the ME and ARH, CSF-borne signals can be redistributed to other sites of action in the brain by the beating of ciliated ependymal cells (Sawamoto et al., 2006). Indeed, in our mice, fluorescent leptin from the CSF reaches not only the hypothalamus but also other downstream target areas known to be leptin-sensitive, such as the hippocampus (Figure S4) (Harvey, 2007). Interestingly, while our recent work shows that ARH tanycytes together with fenestrated ME microvessels themselves constitute a highly plastic blood-hypothalamus barrier that responds to blood-borne metabolic signals by VEGF-A-mediated changes in permeability (Langlet et al., 2013), the beating of ciliated ependymal cells is also controlled by a hypothalamic peptide involved in energy metabolism, melanin-concentrating hormone, released by lateral hypothalamic area neurons (Conductier et al., 2013) efferent to leptin-sensitive ARH neurons (Elias et al., 1998). Thus, fenestrated endothelial cells, ME and “inverted” ARH tanycytess, and ciliated ependymal cells together constitute a highly sensitive “supply chain” for the distribution of blood-borne metabolic signals such as leptin to target brain areas that control behavior.

Our data indicate that EGF treatment can restore leptin signaling and function in obese mice with diet-induced leptin resistance, and hasten weight loss when they return to a normal diet, an effect that may be linked not only to improved central leptin sensitivity but to the reinstatement of hypothalamic sensitivity to other metabolic hormones such as ghrelin (Briggs et al., 2010) and/or of hypothalamic neurogenesis (Li et al., 2012; McNay et al., 2012). Although we used EGF here merely to activate ERK, crosstalk between EGF and ghrelinergic pathways could indeed occur via ERK (Nanzer et al., 2004), and EGF is a well-known modulator of neural stem cell proliferation, migration and differentiation (Kuhn et al., 1997; Sun et al., 2005).

The intriguing concept that tanycytes act as a conduit for peripheral metabolic hormones into the brain and the role of deficient tanycytic LepRb-ERK signaling in the pathophysiology of central leptin resistance hold therapeutic potential not only for obesity but also for cognitive impairments (McEwen, 2007), since leptin is also involved in regulating higher brain functions (Harvey, 2007; Yau et al., 2012). The fact that a CSF-mediated route for blood-borne substances into the brain has been rarely evoked before (for an exception, see Nakai and Naito, 1975) only adds to its interest and potential impact.

Tanycytes could thus constitute the missing link in the loop connecting behavior, hormonal changes, signal transduction, central neuronal activation and finally, behavior again.

EXPERIMENTAL PROCEDURES

Details are provided in the Supplemental Experimental Procedures.

Animals

3-4-month-old male C57Bl/6 mice (Charles River) were provided water and standard chow (Special Diet Services) *ad libitum*. Some mice received a high-fat (60% fat) diet (Research Diets). *LepR*$^{db/db}$ (db/db) mice were bought from Jackson Labs. All studies were approved by the Lille University IACUC.

Assessment of leptin uptake in the ME and MBH

The ME and MBH were microdissected from mice decapitated 5, 15 or 45 min following i.p. injection of leptin (3mg/kg) or vehicle (10mM Tris-HCl), pooled (2 mice/sample) and subjected to western blotting. To rescue leptin transport, EGF (1mg/kg) or vehicle (10mM acetic acid in PBS) was given 15min before sacrifice.

Fluorescent leptin assays

Fluorescent bioactive leptin or leptin antagonist (LAN) (25 nmol/mouse; Cisbio Bioassays) (Vauthier et al., 2013) was injected into the jugular vein of anesthetized mice, and mice sacrificed 5min later to assess tanycytic leptin uptake by fluorescent microscopy.

Leptin-neutralizing antibody delivery

Leptin-neutralizing antibodies (R&D systems) were stereotaxically injected into the 3rd ventricle (anteroposterior -1.7 mm; 0 mm from midline; dorsoventral -5.6 mm) of anesthetized mice; 10min later, leptin was given i.p. in conscious mice, and mice sacrificed 45min later.

Immunofluorescence, immunoprecipitation and western blot analyses

Details are provided in the Supplemental Experimental Procedures.

Primary cell culture experiments

Tanycytes were isolated as described previously (Prevot et al., 2003).

Physiological measurements

Total energy expenditure, O_2 consumption, CO_2 production, food intake and ambulatory movements were measured (Tschop et al., 2012); body weight was used to assess leptin sensitivity.

Statistics

Data were analyzed on GraphPad PRISM (V5.0a) using 1-way or 2-way ANOVA followed by Tukey multiple-comparison tests. Differences between two groups were determined using unpaired two-tailed Student's t-tests. The threshold for significance was $p<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Blood-borne leptin enters the hypothalamus through the median eminence
- Tanycytes act as a checkpoint in the entry of leptin into the brain
- LepRb-ERK signaling is required for hypothalamic leptin uptake
- Triggering ERK signaling in tanycytes rescues leptin function in obese mice

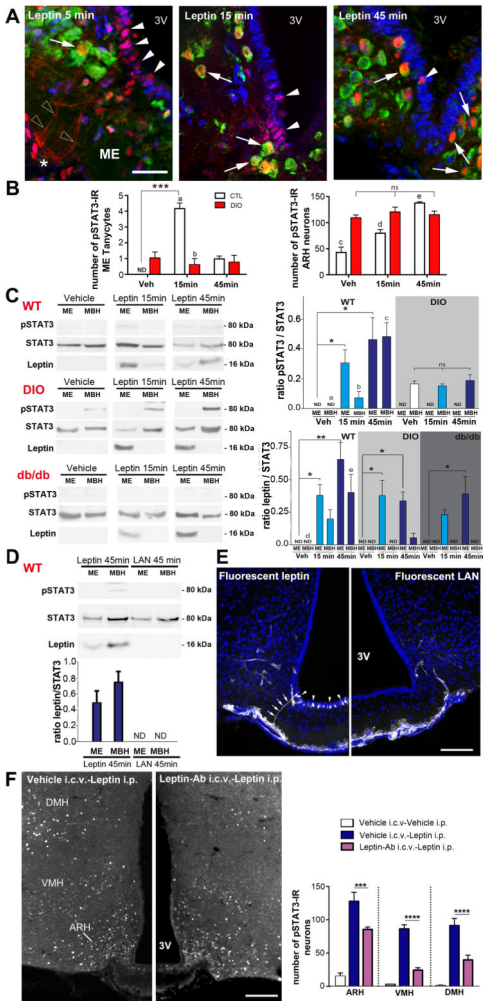

Figure 1. Leptin transport into the MBH via the ME requires LepR signaling and is disrupted in DIO mice

(A) Representative photomicrographs showing pSTAT3-IR (red) in the ME of adult male WT mice 5, 15 and 45 min after i.p. leptin administration (3mg/kg). Arrowheads and empty arrowheads show pSTAT3-IR in tanycyte cell bodies lining the floor of the 3rd ventricle (3V) and tanycytic processes contacting the pial surface of the brain (asterisk), respectively. Arrows show neurons immunoreactive for HuC/D (green) and pSTAT3. (B) Graphs representing the mean number of ME tanycytes and arcuate neurons labeled for pSTAT3 per hemisection, 15 and 45 min after the i.p. administration of leptin (3mg/kg) or vehicle in control (CTL; white bars) and DIO mice (red bars). $p<0.05$ for a *vs.* b and c *vs.* d and e; ns: not statistically different. (C) Representative western blots (WB) and quantitative comparison of phosphorylated and total STAT3 and leptin in ME and MBH explants from adult male WT, DIO and *db/db* mice 15 and 45 min after i.p. leptin (3mg/kg) or vehicle administration (Veh.) (n=3-4 per group). $p<0.001$ for a *vs.* b; $p<0.05$ for d *vs.* e. ND: not detected. (D) Similar experiments as in B performed with WT mice 45 min after the i.p. administration of bioactive leptin or the mutated antagonist LAN (3mg/kg) (n=3-4 per group). (E) Representative photomicrographs showing tanycytic processes (arrows) and cell bodies (arrowheads) labeled by fluorescent leptin (25nmoles/animal; white labeling), but not fluorescent LAN (25nmoles/animal), 5 min after i.v. injection. However, fluorescent LAN labels blood vessels in the external zone of the ME and the ARH, as does fluorescent leptin (asterisk). (F) Representative photomicrographs and quantitative comparison of pSTAT3-IR

(white) in the ARH, VMH and DMH of mice treated i.c.v. with vehicle or leptin-neutralizing antibodies (Leptin-Ab, n=5) 45 min after the i.p. administration of leptin (3mg/kg), or vehicle (n=3-5 per group). Scale bars: 20μm (A), 100μm (E) and 200μm (F). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, ****: $p<0.0001$ leptin vs. vehicle treatment and between indicated groups. Values indicate means ± SEM. See also Figure S1, S2 and S4.

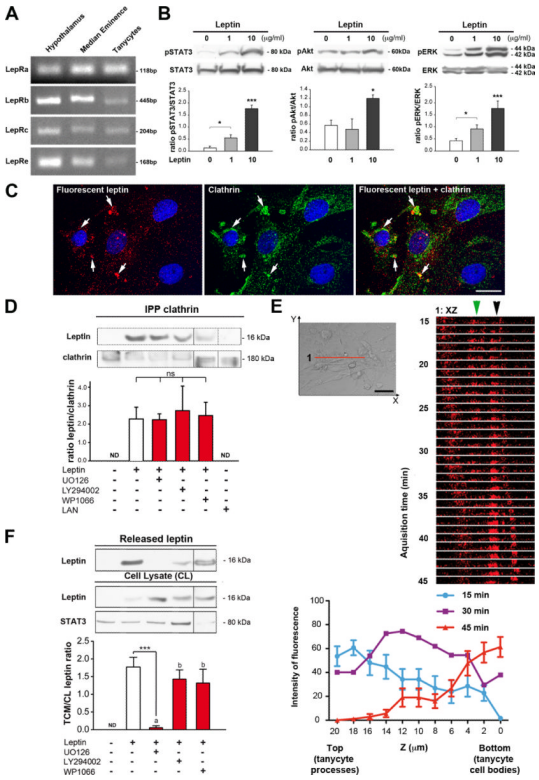

Figure 2. ME Tanycytes express functional LepR, internalize leptin through clathrin-coated vesicles and release captured leptin via an ERK-dependent signaling pathway

(A) RT-PCR analysis of the expression of mRNAs for LepR isoforms in primary cultures of tanycytes. (B) Representative WB and quantitative comparison of phosphorylated and total STAT3, Akt and ERK 15 min after leptin or vehicle treatment of cultured tanycytes (n=4 per group). (C) Representative confocal images for clathrin-IR (green) in tanycytes treated for 15 min with 50nM fluorescent leptin (red). Scale bar: 10µm. (D) Representative WB and quantitative comparison of leptin and clathrin in immunoprecipitated (IPP) clathrin-coated vesicles from tanycytes treated for 15 min with vehicle, LAN (1µg/ml) or leptin (1µg/ml) in the presence or absence of U0126 (10µM), LY294002 (30µM) and WP1066 (30µM). (E) Representative live-cell imaging of fluorescent leptin distribution within tanycytes over an acquisition time of 30 min. *Top left panel*, xy transmission image of the cultured tanycytes that were analyzed. *Right panel*, xz reconstruction of section 1 showing the dynamics of leptin transport from 15 to 45 min following leptin administration. The green and black arrowheads show individual cells that are releasing and taking up fluorescent leptin, respectively. Scale bar: 50µm. (F) Representative fluorescence profile in a single cell 15, 30 and 45 min after treatment. (G) Representative WB and quantitative comparison of leptin in cell lysates from leptin-loaded tanycytes and in the medium (released leptin) over 15 min, in the presence or absence of the pharmacological inhibitors of LepR signaling pathways used in D. STAT3 was used as a loading control for cell lysate samples. $p<0.001$ for a *vs.* b. *$p<0.05$, ***$p<0.001$ treated *vs.* control and indicated groups. Values indicate means ± SEM. See also Figure S3.

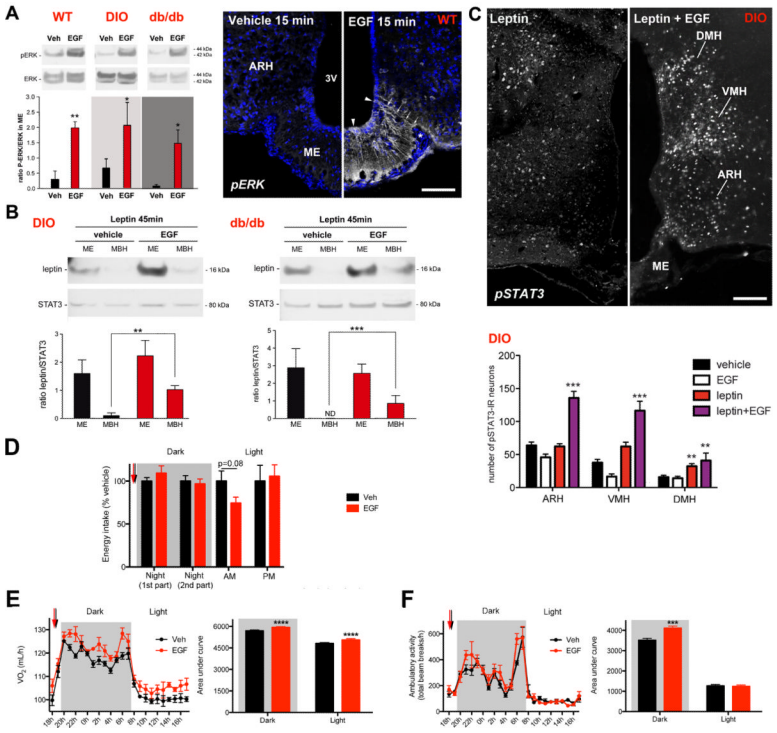

Figure 3. EGF-mediated activation of ERK signaling in the ME restores the transport of leptin into the MBH, increases energy expenditure and promotes locomotor activity in obese mice

(A) EGF (1mg/kg, 15 min) promotes ERK activation in the ME as shown by the quantitative comparison of WB (left panels; n=4-5 per group); right panels: immunofluorescence labeling of pERK (white) in tanycyte cell bodies (arrowheads) and processes (arrows) contacting the pericapillary space (asterisk) in the ME. 3V, 3rd ventricle; ARH, ARH. (B) Representative WB and quantitative comparison of leptin in ME and MBH explants from DIO (n=4) and db/db (n=3) mice after i.p. leptin administration (3mg/kg), with or without EGF treatment 15 min before sacrifice. (C) Representative photomicrographs and quantitation (n=4) of pSTAT3-IR immunofluorescence after i.p. administration of leptin (3mg/kg, 45 min) with or without EGF treatment 15 min before sacrifice. (D-F) Five-day food (D) and oxygen (E) consumption, and locomotor activity (F) monitoring in DIO mice injected once daily with EGF (1mg/kg/day, red) or vehicle (Veh, black) i.p. at 18:00 (n=7 mice per group). Scale bars: 100μm (A) and 200μm (C). ****: p<0.0001; ***: p<0.001; **: p<0.01 EGF and/or leptin vs. vehicle treatment. Values shown are means ± SEM.

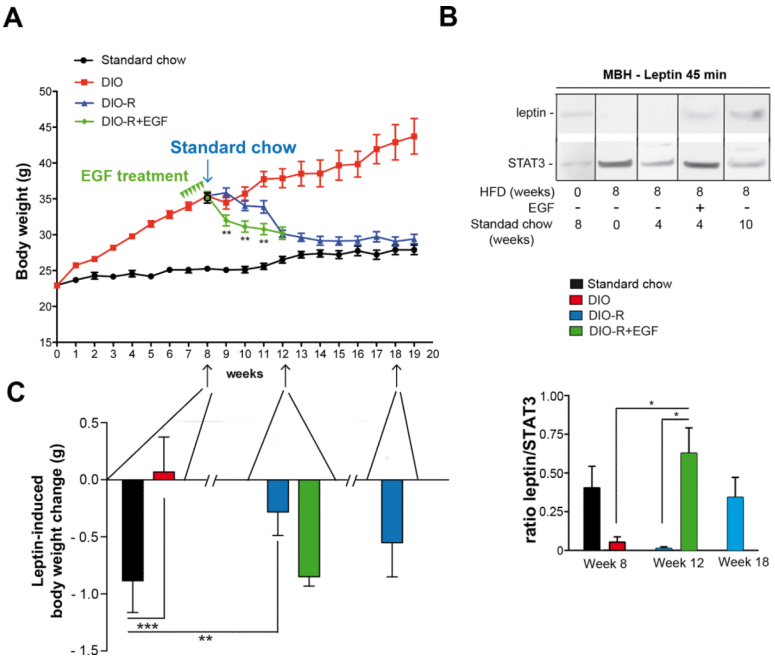

Figure 4. EGF treatment accelerates the restoration of leptin sensitivity in obese mice once they are replaced on a normal diet

(A) Body weight change over 20 weeks in standard-chow-fed mice, DIO mice and DIO mice replaced on a standard diet after 1 week of EGF treatment (1mg/kg/24h; DIO-R+EGF, arrowheads) or no EGF treatment (DIO-R) (n=6-8 per group). (B) Representative WB and quantitative comparison of leptin in MBH explants from standard-chowfed, DIO, DIO-R and DIO-R+EGF mice (n=3-4 per group) after i.p. leptin administration (3mg/kg) at different time points. (C) Body weight change in standard-chow-fed, DIO, DIO-R and DIO-R+EGF mice after daily i.p. leptin administration for 3 days (n=6-8 per group). ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$, EGF *vs.* vehicle treatment and between indicated groups. Values shown are means ± SEM.