Brief Communication

MOLECULAR METABOLISM

Deficiency of leptin receptor in myeloid cells disrupts hypothalamic metabolic circuits and causes body weight increase

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ABSTRACT

Objective: Leptin is a cytokine produced by adipose tissue that acts mainly on the hypothalamus to regulate appetite and energy homeostasis. Previous studies revealed that the leptin receptor is expressed not only in neurons, but also in glial cells. Microglia are resident immune cells in the brain that play an essential role in immune defense and neural network development. Previously we reported that microglial morphology and cytokine production are changed in the leptin receptor deficient *db/db* mouse, suggesting that leptin's central effects on metabolic control might involve signaling through microglia. In the current study, we aimed to uncover the role of leptin signaling in microglia in systemic metabolic control.

Methods: We generated a mouse model with leptin receptor deficiency, specifically in the myeloid cells, to determine the role of microglial leptin signaling in the development of metabolic disease and to investigate microglial functions.

Results: We discovered that these mice have increased body weight with hyperphagia. In the hypothalamus, pro-opiomelanocortin neuron numbers in the arcuate nucleus (ARC) and α-MSH projections from the ARC to the paraventricular nucleus (PVN) decreased, which was accompanied by the presence of less ramified microglia with impaired phagocytic capacity in the PVN.

Conclusions: Myeloid cell leptin receptor deficient mice partially replicate the *db/db* phenotype. Leptin signaling in hypothalamic microglia is important for microglial function and a correct formation of the hypothalamic neuronal circuit regulating metabolism.

Keywords Microglia; Diabetes; Obesity; POMC; α-MSH

1. INTRODUCTION

Leptin is an important adipokine regulating energy balance mainly through signaling in the hypothalamus. Binding of leptin to its receptors on key neuronal populations controlling metabolism in the arcuate nucleus (ARC) inhibits feeding and generates satiety signals [1—3]. Lack of leptin due to a gene mutation (*ob/ob* mouse) results in severe obesity [4] and lack of leptin receptors (*db/db* mouse) produces an obese and diabetic phenotype [5]. In diet induced obesity, leptin resistance of the hypothalamus has been proven to be an important mechanism [6]. Therefore, understanding leptin signaling in the hypothalamus is crucial to shed light onto underlying mechanisms leading to obesity and diabetes.

In the classic view, leptin is assumed to act mainly through leptin receptors (LepR) on hypothalamic neurons, but, more recently, the LepR has also been identified on glial cells [7,8]. To date, the functional significance of LepR in glial cells has not received much attention. Recent studies pointed out that the LepR in astrocytes participates in modulating synaptic input onto hypothalamic neurons [9], suggesting that leptin signaling in glial cells is a crucial part of the hypothalamic leptin signaling mechanism.

Microglia are an important neural subpopulation of the glial cells, responsible for the maintenance of a healthy microenvironment in the brain. LepR has been reported to be expressed in microglia [8,10,11]. In our previous study, we reported that in the hypothalamus of LepR deficient *db/db* mice, genes related to microglial function are modulated, while phagocytic capacity was also significantly reduced [12]. We and others further reported that leptin can directly regulate microglia cytokine production both in a microglial cell line and in cultured primary microglia [8,10—12].

To investigate the significance of microglial leptin signaling in microglial function and central leptin signaling, we generated a *LepR* knockout mouse model specific in myeloid cells including microglia and macrophages, by crossing the *Cx3cr1*-Cre mouse with Leptin

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receptor loxP mouse and determined the role of microglial LepR in the brain control of energy homeostasis.

2. MATERIALS AND METHODS

2.1. Animals

All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the Helmholtz Center Munich, Bavaria, Germany. All mice were group housed on a 12-h light/dark cycle at 23 °C, with free access to food and water. Mice were fed a standard chow diet (LM-485, Teklad).
The myeloid cell specific leptin receptor knock-out mouse line was generated by crossing the leptin receptor loxP mouse line (Jax mice stock no:008327) with *Cx3cr1*-Cre mouse line (Jax mice stock no: 025524) - transgenic mice harboring the Cre recombinase driven by the promoter of chemokine (C-X3-C motif) receptor 1, which is expressed in the mononuclear phagocyte system, including microglia. The knock out mice with leptin receptor loxP sites and Cre are referred to as "*LepR* $fl^{+/+}$ *Cx3cr1* $Cre^{+/-}$". Their littermates with Cre promoter, but without *LepR* loxP insertion, are referred to as "wild type (WT)" in the following text.

2.2. Metabolic phenotyping

Body weight was measured weekly after weaning. Food intake was measured on a daily basis for 5 days at the end of the study. Whole-body composition (fat and lean mass) was measured using nuclear magnetic resonance technology (EchoMRI-100; Echo Medical Systems).

2.3. Glucose tolerance test

An intraperitoneal glucose tolerance test (ipGTT) was performed by injection of D-glucose (2 g/kg, 25% wt/vol in 0.9% wt/vol NaCl) after a 5-h fast from 8 AM. Tail blood glucose levels (mg/dL) were measured with a TheraSense Freestyle glucometer (Abbott Diabetes Care) before (0 min) and at 15, 30, 60 and 120 min after injection.

2.4. Primary microglia culture

Primary microglia cultures were performed as described before [12]. Briefly, brain tissues were isolated from neonatal mice and triturated and seeded in a 175-cm^2 cell culture flasks. Cells were incubated at 37 °C and 5% CO_2 for 9 d with MEM containing 10% FCS and 1% antibiotics. Medium was changed every 3 days. When mixed glial culture reached 90% confluency, L929 cell line conditioned medium was added into the regular MEM (30% v/v) and incubated with cells for 2 days to stimulate microglia proliferation. When microglia became confluent, flasks were placed in a 37 °C shaker at 150 rpm for 1 h to detach microglia. After shaking, medium was collected and passed through 40 um filters. After centrifugation for 5 min at 380 g, the cell pellet was re-suspended in MEM +10% FCS +1% antibiotics and seeded for experiments.

2.5. Immunohistochemistry and immunofluorescence

Immunohistochemistry was carried out as described before [13]. Briefly, mice used for immunohistochemistry were perfused and fixed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4 °C. After being equilibrated for 48 h with 30% sucrose in TBS, coronal sections (30 μm) were cut on a cryostat, and sections were rinsed in 0.1 M TBS. Coronal sections containing the mediobasal hypothalamus (MBH) or paraventricular nucleus (PVN) were incubated with primary antibodies at 4 °C overnight. Sections were rinsed and incubated in biotinylated secondary goat anti-mouse IgG and avidin-biotin complex (Vector Laboratories). The reaction product was visualized by incubation in 1% diaminobenzidine with 0.01% hydrogen peroxide. For immunofluorescent staining, fluorescent secondary antibodies were added accordingly. Primary antibodies: rabbit anti-ionized calcium-binding adapter molecule 1 (iba1) (cat. 234003, Synaptic Systems), rat anti-CD68 (ab53444, Abcam), rabbit anti-neuropeptide Y (NPY) (ab30914, Abcam), rabbit anti-Pro-opiomelanocortin (POMC) (Cat.H-029-30, Phoenix), goat anti-Agouti-related protein (AGRP) (AF634, R&D), rabbit anti-Alpha-Melanocyte Stimulating Hormone (α-MSH) (ab123811, Abcam), rabbit anti-NeuN (24307, Cell signaling).

2.6. Image analysis

Immunohistochemistry images were analyzed by software Image J. Appropriate color threshold for DAB staining for each immunoreactivity (-ir) was set up manually for the first picture and applied to all the rest pictures. The brain area covered by the DAB staining signals (α-MSH and AGRP) above the color threshold of each individual brain section in the fixed hypothalamic region was used to quantify the fiber density. Immunofluorescent images were analyzed by Imaris 8.0 (Bitplane). For CD68 and Iba1 co-staining, the volume of CD68-ir phagosomes per Iba1-ir microglial cell was measured by Imaris 8.0 on each brain section and averaged per animal.

2.7. Statistics

All statistical analysis was performed using GraphPad Prism. Two groups were compared by using two-tailed unpaired Student's t test. P values lower than 0.05 were considered significant. All results are presented as mean ± SEM.

3. RESULTS

3.1. Leptin receptor KO generation in myeloid cells

To reconfirm the existence of the leptin receptor (LepR) in microglia, we examined leptin receptor expression by quantitative PCR in primary cultures of mouse microglia. PCR products were shown on gels (Figure S1A). Next, to investigate the significance of leptin signaling in the microglia, we generated a mouse model with *LepR* specifically knocked out in the mononuclear system (*LepR* $fl^{+/+}$ *Cx3cr1* $Cre^{+/-}$) (Figure S1B). We found that mice without LepR in the myeloid cells gained body weight faster than WT controls at the age of 10wk fed a standard chow diet, and this difference in body weight persisted throughout the study (Figure 1A). The *LepR* $fl^{+/+}$ *Cx3cr1* $Cre^{+/-}$ mice had higher lean mass and showed a trend for a higher fat mass (Figure 1B). Food intake was also higher in the *LepR* $fl^{+/+}$ *Cx3cr1* $Cre^{+/-}$ mice (Figure 1C). Interestingly, glucose tolerance, basal glycemia and non-esterified fatty acid level (NEFA) were not changed in these animals compared to WT mice (Figure 1D–F). We also monitored female mice and they were fertile and did not show a difference in body weight (Figure S1C).

3.2. Microglia are dystrophic in PVN of *LepR* $fl^{+/+}$ *Cx3cr1* $Cre^{+/-}$ mice

To examine the impact of LepR loss in microglia on cellular function, we first analyzed microglial morphology by iba1-ir in various brain regions. We found that microglia with LepR deficiency were less ramified in the PVN, while the microglial cell number did not change (Figure 2A_1, B_1, C, D). Such morphological changes in microglia were limited to the PVN and were not observed in other brain regions including the arcuate nucleus (ARC) and cortex (Figure 2A_2, B_2, A_3, B_3,

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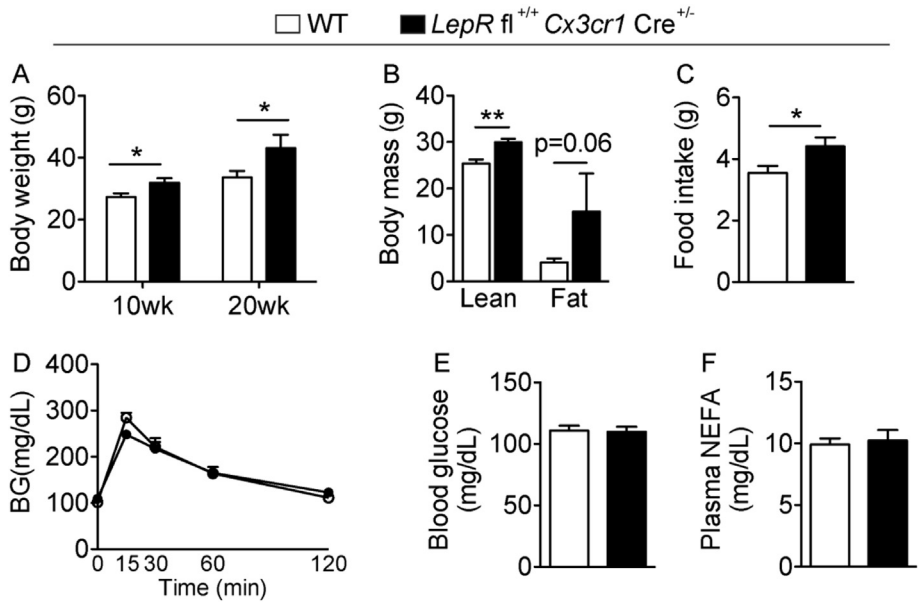

Figure 1: Leptin signaling in microglia is required for metabolic homeostasis. Metabolic phenotypes of *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ male mice are shown, with increased body weight (A), higher lean mass and tendency of increase in fat mass (B), increased food intake (C), and unaltered glucose tolerance (D), basal glycemia (E) and non-esterified fatty acid level (NEFA) (F), compared to WT. Data are presented by Mean ± SEM; *p < 0.05; **p < 0.01.

C, D). The phagocytic capacity of hypothalamic microglia was compared between *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice and WT controls by CD68 and iba1 co-staining. We found that microglia without LepR have less CD68-ir phagosomes (total volume) per iba1-ir microglia (Figure 2E–G), indicating a possible down-regulation of phagocytic capacity. Thus, the current model indicates that leptin signaling might be crucial for microglial phagocytic functions.

3.3. Leptin receptor deficiency in myeloid cells decreases POMC neurons projecting from ARC to PVN

In the hypothalamus of *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice, a significant decrease of POMC-ir neuron number was found in the ARC (Figure 3A$_1$, B$_1$, C), with a trend for increased AGRP-ir compared to WT mice (Figure 3A$_2$, B$_2$, D). The PVN is the most important secondary nucleus receiving projections from the leptin sensitive neurons located in the ARC. In the PVN of the *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice, we found α-MSH-ir fiber density to be significantly reduced (Figure 3E$_1$, F$_1$, G), while AGRP-ir and NPY-ir fiber density was not affected (Figure 3E$_2$, F$_2$, H; Fig. S3A–C). We also found a loss of neurons in the PVN of *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice, as indicated by less NeuN-ir cells (Fig. S3D–F). These data suggest that leptin signaling in microglia is necessary for a proper development of hypothalamic regulatory circuits, especially the POMC neurons projecting from the ARC to the PVN.

Although our mouse model is characterized by a loss of LepR in microglia of the brain, LepR is also absent in peripheral monocytes and macrophages. However, as shown in Fig. S2, gene expression of inflammatory and glucose homeostasis markers was not changed in the *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice. A previous study used the same leptin receptor flox mice crossed with lysozyme M promoter to study the leptin receptor in myeloid cells [14]; this animal model had high knockout efficacy in the peripheral myeloid cells but not in the hypothalamus, and the mice did not develop obesity as our current model, which suggests a microglia specific effect on hypothalamic circuits in our study.

4. DISCUSSION

In this study, we confirmed the presence of functional leptin receptors in microglia and showed that microglial *LepR* knock out mice have higher body weight with hyperphagia. In the hypothalamus, we found less POMC-ir neurons in the ARC and less α-MSH-ir fibers in the PVN, accompanied with neuron loss. Finally, microglia in the PVN was less ramified and showed impaired phagocytic capacity.

Our previous findings of abnormal microglial phenotypes in *db/db* mice led us to investigate the role of microglial leptin receptors in the central leptin signaling mechanism. Compared with *db/db* mice, *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice partially replicated *db/db* phenotypes, but in a less severe form. *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice gained body weight faster with increased food intake but did not become diabetic. *Db/db* mice had low POMC and high AGRP expression in the ARC [15], with a reduction in both α-MSH-ir and AGRP-ir fibers [16]. In *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice, we observed less POMC-ir neurons in the ARC and less α-MSH-ir fibers in the PVN. AGRP immunoreactivity tended to increase in the ARC, but showed no changes in the PVN. The similarities between the phenotypes of *db/db* and *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice indicate that microglial leptin signaling is part of the central leptin signaling mechanism.

The deficiency of the ARC-PVN projection has been observed in the *ob/ob* mice as well and could be rescued by leptin injections at an early postnatal stage, demonstrating the trophic action of leptin on the formation of the hypothalamic feeding circuit during development [17,18]. Our data indicate that such a trophic effect of leptin on the hypothalamic neuronal circuits might involve leptin signaling in the microglia. Microglia are known to play an important role in the synaptic pruning progress by engulfing the redundant synapses in order to form a correct neural network [19,20]. This pruning function is dependent on the microglial phagocytic capacity. In our previous study, we reported that CD68-ir, a phagocytic indicator of microglia, is reduced in *db/db* mice [12]. In the microglial specific *LepR* knockout model

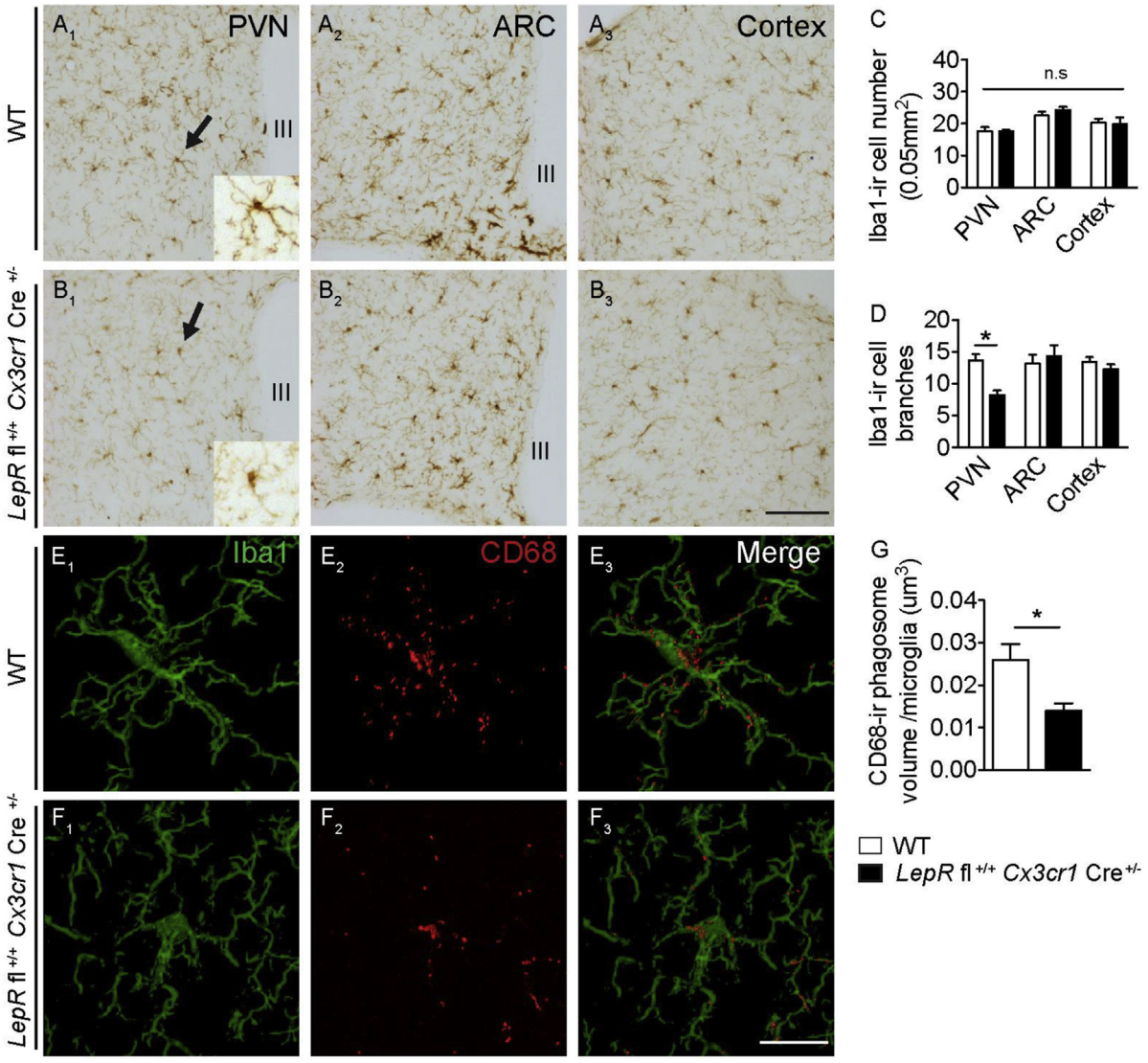

Figure 2: Microglia in *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice have dystrophy in the PVN. Iba1-ir positive microglia number is unaltered in the PVN (A_1, B_1, C), ARC (A_2, B_2, C) and cortex (A_3, B_3, C). However, the branches (ramifications) of the microglia in the PVN are reduced in *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice compared to WT (A_1, B_1, D), which was not found in the ARC (A_2, B_2) or cortex (A_3, B_3); the high magnification of microglial cell pointed by dark arrow is displayed at right bottom of A_1 and B_1. The phagocytic capacity of microglia in the PVN is also decreased in *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ ($F_{1\text{-}3}$) compared to the controls (E_{1-3}), indicated by less CD68-ir phagosome volume in each microglia (G). Data are presented by Mean $\pm$ SEM; *p $<$ 0.05. Scale bar: 100 um in A, B; 20 um in E & F. III: Third ventricle. All data presented are from male mice.

presented here we also observed reduced CD68-ir phagosomes in microglia in the PVN, together with neuron loss and less α-MSH-ir fibers. Therefore, we propose that leptin signaling is important for microglial phagocytic capacity and its synaptic pruning function in the mediobasal hypothalamus, which is required for the formation of efficient hypothalamic neuronal circuits during development.

The morphological changes of microglia in the PVN of *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice might be caused directly by the microglial leptin receptor deficiency, but also might be the consequence of a reduced reactivity of the surrounding neurons. We found less ramified microglia in the PVN, but not in other brain regions of the hypothalamus, perhaps due to the more severe neuronal network disruption in the PVN. Microglia and neurons have reciprocal interactions through cytokines, neuropeptides, and neurotransmitters [21]. Microglia also express melanocortin-4 receptors [22—24], gamma-aminobutyric acid (GABA) receptors [25], and glutamate receptors [26,27]. Thus, microglial reactivity could be regulated by either α-MSH, GABA or glutamate released from ARC neuron terminals in the PVN [28,29]. Therefore, microglial LepR deficiency might interrupt neuronal network development in the MBH, and the reduced neuronal activity might feedback to microglia in downstream area innervated by MBH neurons. To further study this question, an inducible *LepR* knockout model would be helpful to address the function of microglial LepR at different life stages.

AUTHOR CONTRIBUTIONS

Y. G. generated the mouse model, performed the metabolic phenotyping, primary cultures, microglia isolation, and western blot, and constructed the manuscript; A. V. I. performed the immunohistochemistry and analyzed the images; I. M., N. L. K., and M. J. K analyzed the plasma parameters and tissue gene expression; R. Z. T.

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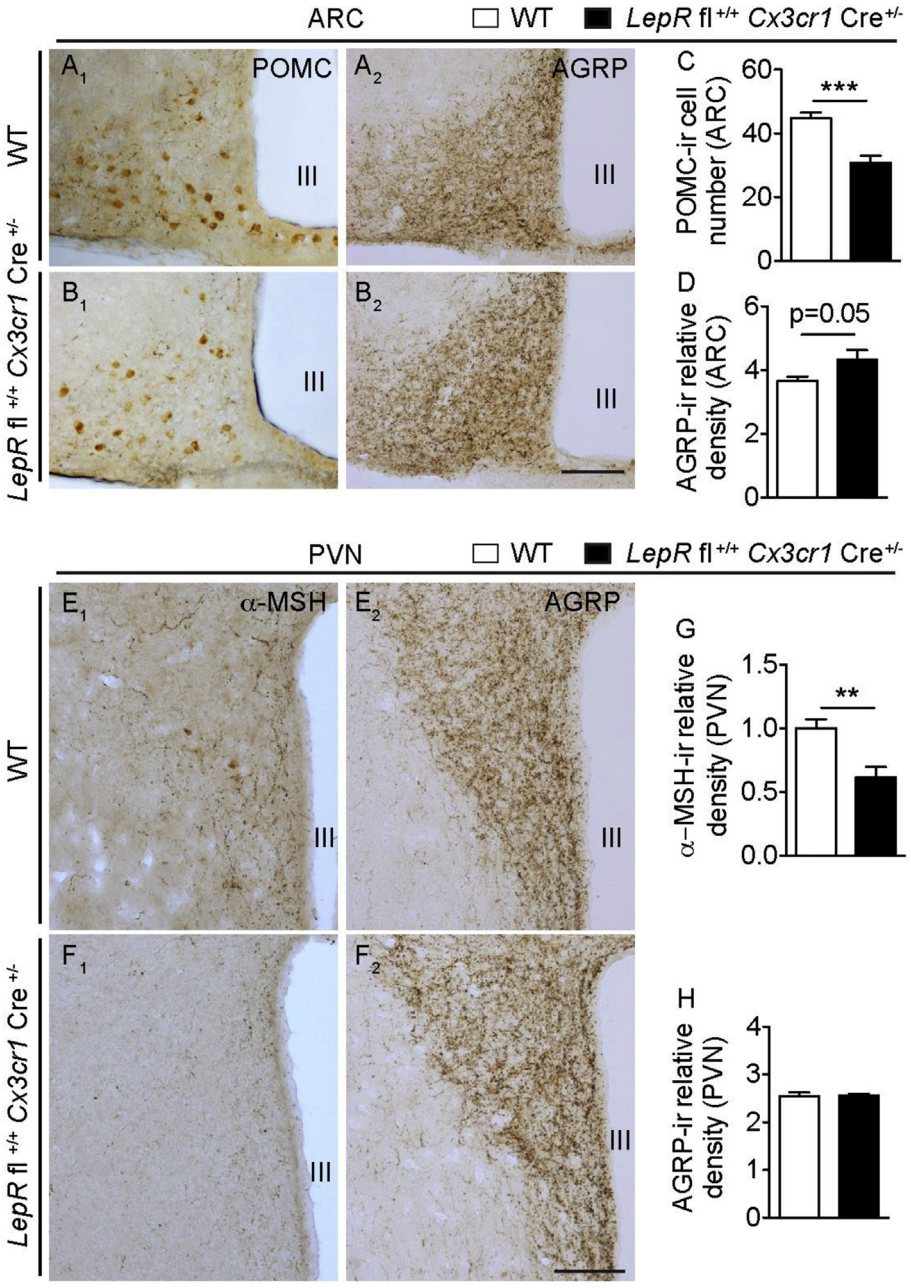

Figure 3: *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice have less POMC neurons and less projections from the ARC to PVN. *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice (B_1) have a lower POMC cell number compared with WT (A_1, C) and a tendency towards increased AGRP-ir (B_2) compared with WT (A_2, D) in the ARC. Meanwhile, the projections from the ARC to PVN containing α-MSH are decreased in the PVN in *LepR* fl$^{+/+}$ *Cx3cr1* Cre$^{+/-}$ mice (F_1) compared with WT (E_1, G), while AGRP fiber density were not altered in the PVN (E_2, F_2, H). Data are presented by Mean ± SEM; **p < 0.01, ***p < 0.001. Scale bar: 100 um. III: Third ventricle. All data presented are from male mice.

helped with the microglia isolation; A. K. and S. M. H. drafted the manuscript. C. X. Y. designed the study, supervised the experiments, interpreted the findings and drafted the manuscript.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2017.11.003.

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