

Supplemental Information

Lipoprotein Lipase Maintains Microglial

Innate Immunity in Obesity

Yuanqing Gao, Andrés Vidal-Itriago, Martin J. Kalsbeek, Clarita Layritz, Cristina García-Cáceres, Robby Zachariah Tom, Thomas O. Eichmann, Frédéric M. Vaz, Riekelt H. Houtkooper, Nicole van der Wel, Arthur J. Verhoeven, Jie Yan, Andries Kalsbeek, Robert H. Eckel, Susanna M. Hofmann, and Chun-Xia Yi

Supplemental Information

Lipoprotein lipase maintains microglial innate immunity in obesity

Yuanqing Gao, Andrés Vidal-Itriago, Martin J. Kalsbeek, Clarita Layritz, Cristina García-Cáceres, Robby Zachariah Tom, Thomas O Eichmann, Frédéric M. Vaz, Riekelt H Houtkooper, Nicole van der Wel, Arthur J. Verhoeven, Jie Yan, Andries Kalsbeek, Robert H. Eckel, Susanna M. Hofmann, Chun-Xia Yi

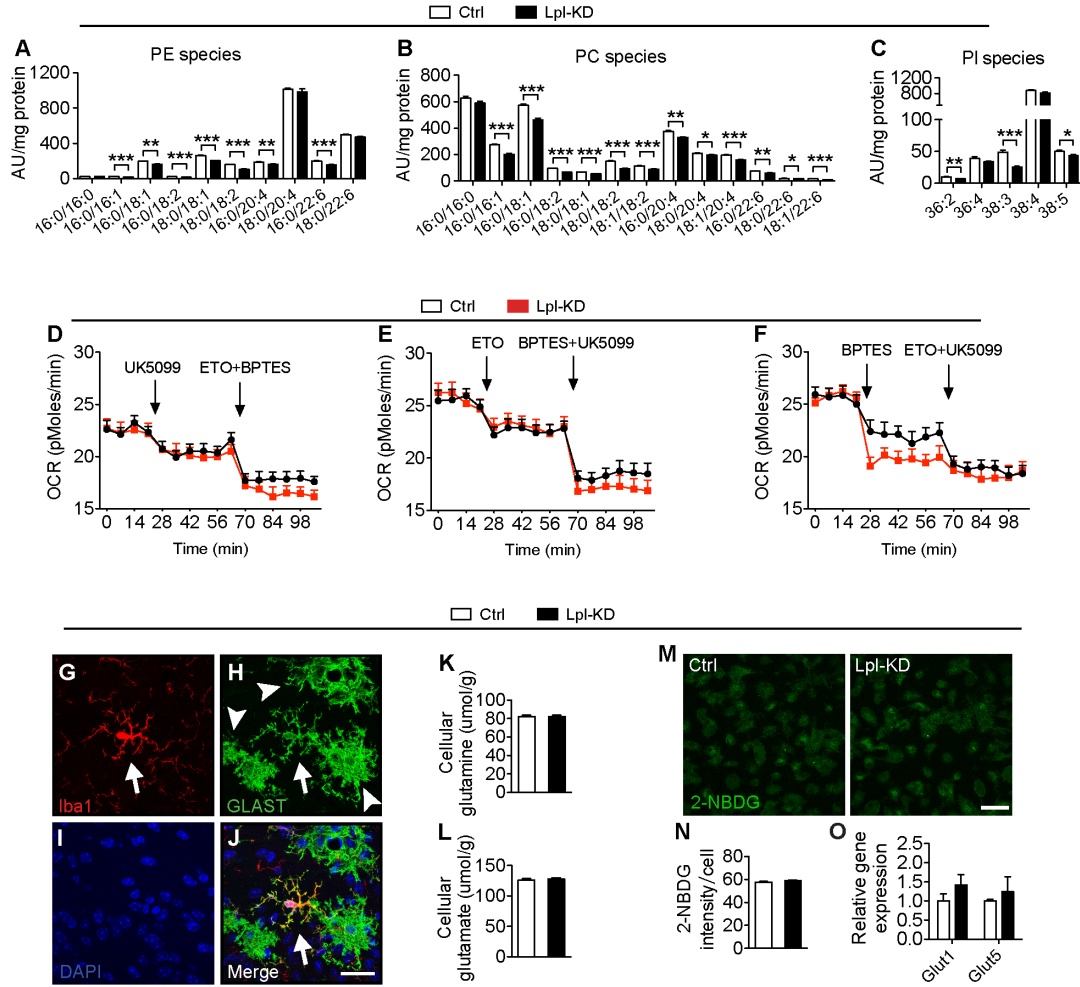


Figure S1. *Lpl*-KD microglial phospholipids contents and fuel utilizations. Related to Figure 1. (A-C) Control and *Lpl*-KD primary microglia phospholipids profiles, including glycerophosphoethanolamines (PE) species (A), glycerophosphocholines (PC) species (B) and glycerophosphoinositols (PI) species (C), $n=4$ per group, AU: arbitrary units. (D-F) Fuel Flex test on glucose (D), fatty acids (E) and glutamine (F) dependency in *Lpl*-KD microglia. UK-5099: 2-Cyano-3-(1-phenyl-1H-indol-3-yl)-2-propenoic acid, BPTES: *N,N'*-[Thiobis(2,1-ethanediyl-1,3,4-thiadiazole-5,2-diyl)] bisbenzeneacetamide, ETO: Etomoxir. (G-J) Iba1-ir microglial cells (pointed by arrows in G, H and J) express GLAST gene (visualized by eGFP in GLAST^{CreERT2}-tdTomato-eGFP mice received tamoxifen injection), notice the GLAST-eGFP in the astrocytes near the Iba1-ir microglia, pointed by arrowheads in H). (K&L) Intracellular glutamine and glutamate concentration do not differ between primarily cultured control microglia and *Lpl*-KD microglia. (M&N) 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose (2-NBDG) uptake do not differ between primarily cultured control microglia and *Lpl*-KD microglia, $n=8-9$ per group. (O) Gene expression of *Glut1* and *Glut5* in microglia isolated from control and microglia-*Lpl*-KD mouse brains are similar, $n=4$ per group. Data are presented by mean \pm SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Unpaired t-test was performed in all experiments. Scale bar: 25 μ m in G-J, 20 μ m in M.

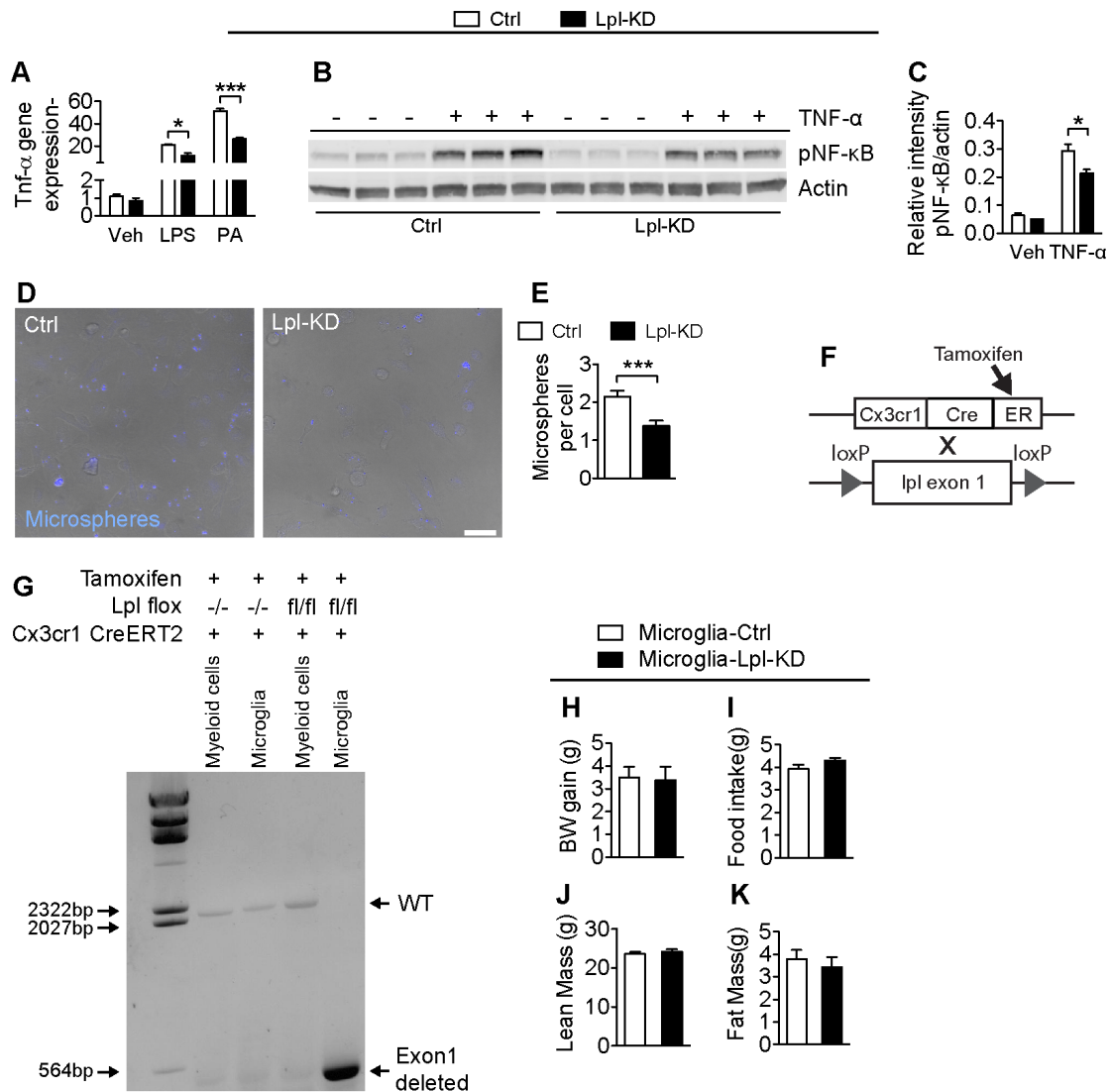


Figure S2. Immune reactivity of *Lpl*-KD microglia and metabolic phenotype of control and microglia-*Lpl*-KD mice on standard chow diet. Related to Figure 1 and Figure 2.

(A) *Tnf-α* gene expression in *Lpl*-KD microglia is significantly less than in control microglia in response to lipopolysaccharides (LPS) and palmitic acid (PA) stimulation. (B&C) Immunoblot of phosphor-NF-κB in response to TNF-α is significantly lower in *Lpl*-KD microglia. (D&E) The uptake of microspheres by *Lpl*-KD microglia is significantly down regulated. (F) Mice breeding strategy for postnatal deletion of *Lpl* gene specifically from microglia. (G) Four weeks after tamoxifen injection, recombinant DNA sequence after *Lpl* exon1 deletion can be detected in microglia isolated from microglia-*Lpl*-KD mouse brain, but not detectable in control mouse microglia, neither in peripheral mononuclear myeloid cells isolated from control mice nor microglia-*Lpl*-KD mice. (H-K) Metabolic phenotypes do not differ between control and microglia-*Lpl*-KD mice fed on standard chow diet, shown by (H) bodyweight gain (at 20 weeks of the age), (I) averaged daily food intake, (J) lean mass and (K) fat mass, n=4-6 per group. Data are presented by mean ± SEM. * $p < 0.05$, *** $p < 0.001$. Scale bar: 30 μm in D.

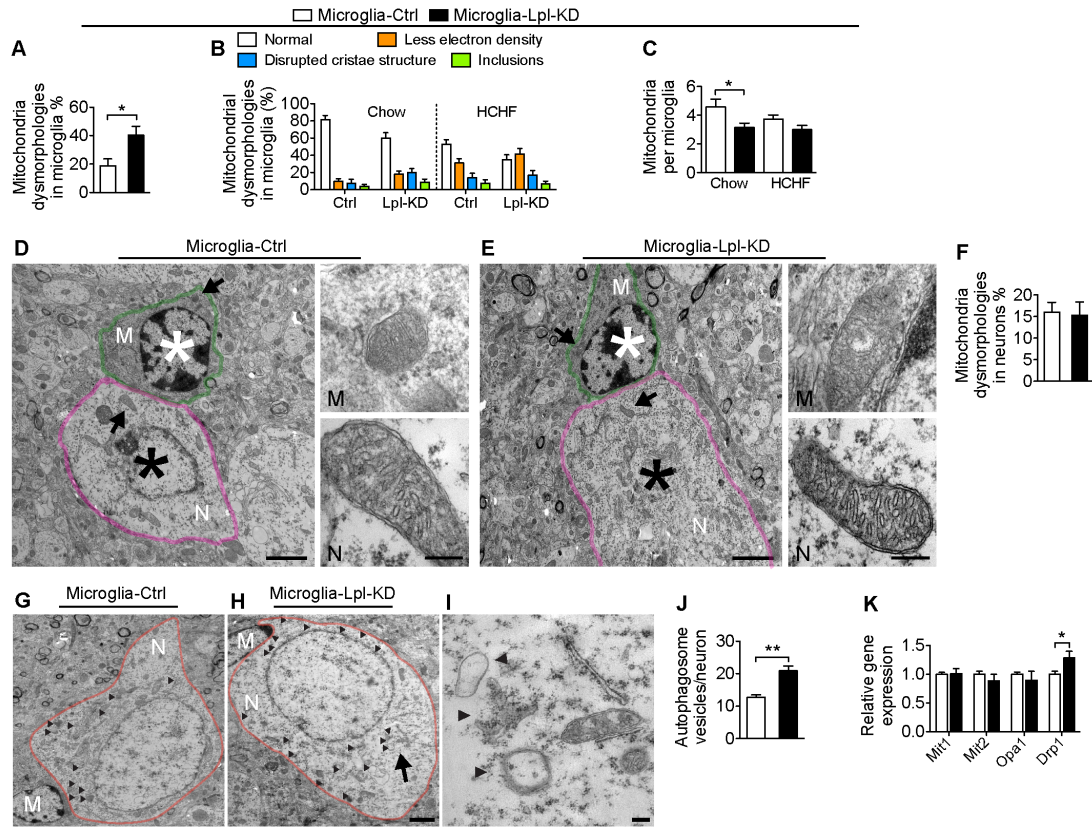


Figure S4. Dysmorphic mitochondria increase in microglia but not in nearby neurons in the mediobasal hypothalamus of microglia-*Lpl*-KD mice on standard chow diet. Related to Figure 4. (A) Dysmorphic mitochondria ratio in microglia in MBH is significantly higher in microglia-*Lpl*-KD mice than in control mice on standard chow diet. (B) Dysmorphic mitochondria ratio classified by lower electron density, disrupted cristae structure and inclusions in microglia in the MBH of microglia-*Lpl*-KD mice on both standard chow diet and HCHF diet. (C) Visible mitochondrial number per microglia is less in microglia-*Lpl*-KD mice MBH on standard chow diet, and tend to decrease on HCHF diet. (D & E) Ultrastructure of mitochondria in microglia (M, outlined by green line) and adjacent neuron (N, outlined by pink line) in the MBH of control mice (D) and microglia-*Lpl*-KD mice (E) on standard chow diet. High magnifications of dark arrows pointed mitochondrion in each cell are amplified in the right panels. *: Nucleus of each cell. (F) Dysmorphic mitochondria ratio in neurons adjacent to microglia in MBH is similar between microglia-*Lpl*-KD mice and control mice on standard chow diet. (G,H&J) Autophagosomes (pointed by black arrow head) in MBH neurons (N, outlined by red line) nearby the *Lpl*-KD microglia (M) (n=10 neurons) are significantly more than in control mice (n=8 neurons). The area pointed by arrow in H is amplified in I. Data are presented by mean \pm SEM. * $p < 0.05$. ** $p < 0.01$. Scale bar: (K) Drp1 gene expression is higher in primarily cultured *Lpl*-KD microglia. Data are presented by mean \pm SEM. Unpaired t-test was performed in all experiments. Scale bar: left panel of D and E: 2 μ m, G and H: 2 μ m, right panel of D and E: 200 nm, I: 200 nm.

Supplemental experimental procedures

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. POMC-eGFP and NPY-eGFP mouse strains were from Jackson Laboratory breeding on C57BL/6 background. For visualizing glutamate aspartate transporter (GLAST)-eGFP, GLAST^{CreERT2} - tdTomato-eGFP mice were generated and injected with tamoxifen as previously described (Garcia-Caceres et al., 2016).

All mice were group housed on a 12/12-hours light dark cycle at 23°C, with free access to food and water. Mice were fed either a standard chow diet (LM-485, Harlan Teklad), or a high carbohydrate high fat (HCHF) diet of which 58% kcal was fat (D12331; Research Diets, New Brunswick, NJ) for 10 weeks from the age of 10 week. Mice fed on a HCHF diet for 10 weeks were used for co staining with iba1.

Metabolic phenotyping of control and microglia-*Lpl*-KD mice

Bodyweight and food intake were monitored throughout the whole study. Food intake was calculated on a weekly basis. Whole-body composition (fat and lean mass) was measured using nuclear magnetic resonance technology (EchoMRI-100; Echo Medical Systems). RER, locomotor activity and heat production were measured by a customized indirect gas calorimetric system at week 10 after tamoxifen injection (TSE Systems GmbH, Bad Homburg, Germany). In short, chow diet or HCHF diet-fed mice were single housed in metabolic cage equipped with PhenoMaster module for indirect gas calorimetry for three days for adaptation, afterward, a continuous 24 hours of measurements of oxygen consumption (VO₂) and carbon dioxide production (VCO₂) were used for calculating respiratory exchange rate, heat production and substrate utilization (fat reserves vs. carbohydrate catabolism). Heat production was normalized by lean body mass.

Primary microglia culture

Brain tissues were isolated from neonatal mice and triturated in MEM (Life Technologies, CA) containing 1% penicillin-streptomycin, 10% fetal calf serum (FCS; life Technologies) and 5.5 mM glucose. The cell suspension was centrifuged for 5 min at 1000 rpm and the pellet was re-suspended and seeded in a 175-cm³ cell culture flasks. Cells were incubated at 37°C and 5% CO₂ for 9 days with MEM containing 10% FCS and 1% antibiotics. Medium was changed every 3 days. When mixed glial culture reach 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 become confluent, flasks were placed in a 37°C shaker at 150 rpm for 1 hour to detach microglia. After shaking, medium was collected and passed through 40 µm 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. For lipopolysaccharides (LPS) and palmitic acid treatment, primary microglia were incubated with serum-free MEM medium with 1 µg/ml LPS or 50 µM palmitic acid (BSA conjugated) for 16 hours, serum-free MEM medium was used as the vehicle control. After 3 times wash with PBS, cells were harvested for analysis.

Microglia isolation from adult brain tissue

For hypothalamic microglial isolation, mice were decapitated and brains were isolated, hypothalami were dissected out according to the mouse brain atlas (Paxinos and Franklin 2008). Fresh hypothalamic tissues were gently homogenized in RPMI (21875-034, Gibco) medium in the hand homogenizer. After 5 min centrifugation at 380 g at 4 degrees, cells were re-suspended with 7 ml RPMI and mixed with 3 ml 100% Percoll solution (p1644, sigma). Cell suspension was then layered slowly onto the top of 2 ml 70% Percoll solution in a new 15 ml falcon and centrifuged at 500 g speed for 30 min at 18 degrees, with accelerate and break rate at 1. After centrifugation, the fuse interphases were transferred into a new 15 ml falcon with 8 ml HBSS (14170112, Gibco) and centrifuged at 500 g for 7 min again. Cells were then incubated with CD11b antibody coated microbeads (130-097-059, Miltenyl Biotec) for 30 min at 4 degrees. CD11b positive microglia were further purified by passing through MACS Columns (130-042-201, Miltenyl Biotec) by magnetic separation.

Blood mononuclear myeloid isolation

Circulating mononuclear cells were isolated from the blood collected from Ctrl or *Lpl*-KD mice. Red blood cells were removed by red blood cell lysis solution (130-94-183, Miltenyl Biotec). After centrifuging, the rest of the cells were re-suspended in 5 ml RPMI medium and layered on top of 5 ml Ficoll (17-1440-02, GE healthcare) solution slowly in a new 15 ml falcon. After centrifuging with 400

g at 20 degrees for 30 min (break 1/1), the fuse interphase was transferred into a new 15 ml falcon and filled up with 8 ml HBSS. After 5 min centrifugation with 200 g at RT, the cells were seeded into culture plate with RPMI. After 30 min, floating cells were washed away and attached mononuclear cells were harvested for PCR analysis.

Glucose Tolerance Test

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

Lipid and Glucose uptake assay

To determine the lipid uptake capacity, 5 ug/ml Dil labeled VLDL particles were incubated with primary microglia in the culture for 1 hour at 37 degrees. Cells were washed with PBS for 3 times and fixed with 1% paraformaldehyde for 10 min before confocal imaging.

To determine the glucose uptake capacity, 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose (2-NBDG) was incubated with primary microglia for 10 min at 37 degrees with a concentration of 10 μ M. Cells were washed with PBS for 3 time and kept in PBS for live imaging by confocal microscope.

Fuel flex assay

Microglia were seeded in an XF96 plate with 100,000 cells per well (Seahorse Bioscience, North Billerica, MA, USA). After 4-Hydroxytamoxifen treatment for 5 days, cells were washed with PBS and incubated with XF assay medium containing 5.5 mM glucose, 2 mM glutamine and 1 mM pyruvate for 1 hour in a 37°C air incubator. The XF96 plate was then transferred to a temperature-controlled (37°C) extracellular flux analyzer (Seahorse Bioscience) and subjected to an equilibration period. Each assay cycle consisted of a 3-min mix and 3-min measure period. Oxygen consumption rate was recorded during the assay to determine the cellular respiration status. Microglia fuel dependency was tested by fuel flex test kit (Seahorse bioscience). All the compounds were included in the kit and diluted with XF assay medium. Assay details were described in the kit. Briefly, after 4 basal assay cycles, 3 inhibitors were added in different order by automatic pneumatic injection to test the fuel dependency of the cells. To determine glucose dependency, UK-5099 (2-Cyano-3-(1-phenyl-1H-indol-3-yl)-2-propenoic acid) was injected from port A to inhibit pyruvate-dependent O₂ consumption. After 6 assay cycles, BPTES and Etomoxir were injected from port B to inhibit glutaminase and long chain fatty acids oxidation. To determine glutamine dependency, BPTES was injected from port A and UK-5099, Etomoxir were injected from port B. To determine fatty acids oxidation, Etomoxir was injected from port A, BPTES and UK-5099 were injected from port B.

Microglia viability assay

To examine the glutamine dependency of *Lpl*-KD microglia, microglia survival rates were measured by CellTiter-Fluor™ Cell Viability Assay (G6080, Promega) with glutamine deprivation medium or glutaminase inhibitor BPTES treatment for 4 hours in control or *Lpl*-KD primary cultured microglia.

Phagocytosis assay

Microspheres (1 μ m, Fluoresbrite® BB Carboxylate, poly science) were coated with PBS-containing 10%FBS at 37 °C for 1 hour. Coated beads were centrifuged and re-suspended in PBS. Coated microspheres were added into the primary cultured control or *Lpl*-KD microglia (1000 microspheres per cell), 1 hour later, cells were washed 3 times with PBS and fixed by 4% paraformaldehyde for confocal imaging. Microspheres per cell were manually counted in each well, 5 wells per group.

Gene expression analysis

For gene expression analysis, hypothalamic tissue was harvested and total RNA was isolated by an RNeasy lipid tissue kit (Qiagen, Germany). After reverse transcription by a QuantiTect Rev. Transcription Kit (Qiagen, Germany), gene expression was analyzed by a real-time PCR with either Taqman probes (Applied Biosystems) and or SYBR primers. *Lpl*: Mm00434764_m1; *Glul*: Mm00725701_s1; *TNF- α* : Mm00443258_m1; *Cd68*: Forward 5'-CTTGTGTTTCAGCTCCAAGCC-3'; Reverse: 5'- GATGGGTACCGTCACAACCT-3'. *Mit1*: Forward 5'-GGATAAAGTCCTCCCCAGCG-3'; Reverse: 5'- GCATGGGCCAGCTGATTAAC-3'. *Mit2*: Forward 5'- GGACCTCCATGGGCATTCTT-3'; Reverse: 5'- CTGTAGCTTCTCACTGGCGT-3'. *Opal*: Forward 5'-GGACCTCCATGGGCATTCTT-3'; Reverse: 5'- GCTTCCGCAGCTCTTTGTTC-3'. *Drp1*:

Forward 5'-TGACCCTGCCACATGGAAAA-3'; Reverse: 5'-TGGATTGGCTCAGGGCTTAC-3'. Hypoxanthine phosphoribosyltransferase 1 (HPRT) was used as a housekeeping gene (Mm01545399_m1 for taqman assay or following primer set for SYBR: Forward 5'-GCAGTACAGCCCCAAAATGG-3'; Reverse: 5'-AACAAAGTCTGGCCTGTATCCAA-3').

For PCR to detect *lpl* delta band after Cre mediated recombination, sequences of primers were set as follows: Forward 5'-CGCCCTGGAACATCACTAAT-3'; Reverse: 5'-CTTCTCAATTGTGGGCAGGT-3'. WT band is of about 2000 bp, delta band is 409 bp.

Western blot

Tissues from MBH of each experimental group were snap frozen. Cells were collected by scraping and sonication with RIPA buffer (R0278, sigma). Protein concentration was determined by BCA assay. The same amount of protein from lysates was placed in 1.5 mL tubes. 4x NuPAGE LDS Sample Buffer (invitrogen, cat.NP0007) was added to each and incubated at 95°C for 5 min. After heating, the mixture was kept on ice for 20 min. 20 ug protein lysates from each sample were then separated by 10% precast gel (Bio-Rad cat.400096180), and transferred to nitrocellulose membranes (Bio-Rad cat.170-4159). After the transfer, the membranes were blocked in 5% milk for 1 hour. Primary antibodies were diluted in 5% milk and incubated with the membrane overnight at 4°C. (Rabbit anti pNF-κB, cell signaling, cat.3033; Rabbit anti-beta-actin, cell signaling, cat.4970); On the following day, membranes were washed by Tris-buffered saline with Tween (TBST) three times for 10 min and incubated with the HRP conjugated secondary antibody for 1 hour at room temperature and washed again in TBST (three times for 10 min). Membranes were then developed by ECL (Bio-Rad, cat.170-5060) and imaged with Odyssey imaging system (LI-COR Bioscience)

Targeted lipidomic analysis

Total lipids of primary microglia (~200 µg protein) were extracted twice according to Folch et al. (Folch et al., 1957) using chloroform/methanol/water (2/1/0.6, v/v/v) containing 500 pmol butylated hydroxytoluene, 1% acetic acid, and 100 pmol of internal standards (ISTD, 17:0-17:0 PC, 19:0-19:0 PC, 17:0-17:0 PE, 17:0-17:0 PS, 17:0-17:0 PG, Avanti Polar Lipids) per sample. Extraction was performed under constant shaking for 60 min at room temperature (RT). After centrifugation at 1,000 x g for 15 min at RT the lower organic phase was collected. 2.5 ml chloroform was added to the remaining aqueous phase and the second extraction was performed as described above. Combined organic phases of the double-extraction were dried under a stream of nitrogen and resolved in 150 µl methanol/2-propanol/water (6/3/1, v/v/v) for UPLC-TQ analysis. Chromatographic separation was modified after Knittelfelder et al. (Knittelfelder et al., 2014) using an AQUITY-UPLC system (Waters Corporation), equipped with a Kinetex C18 column (2.1x50 mm, 1.7 µm; Phenomenex) starting a 15 min gradient with 100% solvent A (MeOH/H₂O, 1/1, v/v; 10 mM ammonium acetate, 0.1% formic acid). A EVOQ Elite™ triple quadrupole mass spectrometer (Bruker) equipped with an ESI source was used for detection. Lipid species were analyzed by selected reaction monitoring (PC: MH⁺ to m/z 184, 25 eV, PE: MH⁺ to -m/z 141, 20 eV, PI: M-H to FA⁻, 50 eV). Data acquisition was done by MS Workstation (Bruker). Data were normalized for recovery and extraction- and ionization efficacy by calculating analyte/ISTD ratios.

Immunohistochemical and immunofluorescent stainings

Immunohistochemistry was carried out as described before (Gao et al., 2014). Briefly, mice used for immunohistochemistry were perfused and fixed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C. After equilibrated for 48 hours with 30% sucrose in TBS, coronal sections (30 µm) were cut on a cryostat and sections were rinsed in 0.1 M TBS. For staining, 3 coronal sections containing the mediobasal hypothalamus (MBH) per mouse were incubated with primary antibodies at 4°C overnight. Sections were rinsed and incubated in biotinylated secondary antibody and avidin-biotin complex (ABC method, Vector Laboratories, Inc., Burlingame, CA). 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-iba1 (cat. 234 003, Synaptic Systems), rat anti-CD68 (ab53444, Abcam), rabbit anti-POMC (H-029-30, Phoenix).

Electron microscopy

Brains were perfused and preserved in 4% paraformaldehyde with 0.2% glutaraldehyde. Coronal sections were obtained by a mouse brain slicer matrix. Arcuate nucleus tissue blocks of 1mm³ were dissected and carefully washed in 0.1 M cacodylate washing buffer. Samples were post-fixed in 1% Osmium tetroxide solution and subsequently incubated overnight in 1.5% uranyl acetate solution.

Samples were extensively washed with Milli-Q water and then dehydrated with increasing concentrations of Ethanol (70, 80, 90 and 100%). Then the samples were incubated in Propylene oxide and later embedded in increasing concentrations of epon mix in propylene oxide. Finally, samples embedded in pure epon were placed in blocks for hardening at 60°C. Ultrathin sectioning was conducted using an Ultra Microtome Leica UC6. Several ultra-thin sections of 70 nm were cut and then collected in grids. Grids were contrasted with uranyl acetate and lead citrate prior to imaging. Images were obtained using a FEI Tecnai T12 transmission electron microscope at 100 kV.

Microglia, which could be identified by dense and highly heterochromatin nucleus (Sobaniec-Lotowska, 2005), were imaged at 11000x, 23000x and 68000x magnification for posterior analysis. For each group, at least 20 microglia and adjacent neurons were imaged and quantified. Peri-nuclear mitochondrial ultrastructure in microglia and neurons were analyzed based on following morphological criteria: integrity of inner and outer membranes, cristae structure and electron density of matrix. Autophagosomes in neurons were characterized by double-membrane bound vesicles.

To quantify the Golgi apparatus size, averaged cisternae thickness of each Golgi complex was measured by total Golgi complex width divided by the cisternae number in that Golgi complex. Data were presented as averaged cisternae thickness per cell.

References

- Folch, J., Lees, M., and Sloane Stanley, G.H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226, 497-509.
- Garcia-Caceres, C., Quarta, C., Varela, L., Gao, Y., Gruber, T., Legutko, B., Jastroch, M., Johansson, P., Ninkovic, J., Yi, C.X., et al. (2016). Astrocytic Insulin Signaling Couples Brain Glucose Uptake with Nutrient Availability. *Cell* 166, 867-880.
- Knittelfelder, O.L., Weberhofer, B.P., Eichmann, T.O., Kohlwein, S.D., and Rechberger, G.N. (2014). A versatile ultra-high performance LC-MS method for lipid profiling. *J Chromatogr B Analyt Technol Biomed Life Sci* 951-952, 119-128.
- Paxinos, G., and Franklin, K. (2008). *The Mouse Brain in Stereotaxic Coordinates*. Elsevier.
- Sobaniec-Lotowska, M.E. (2005). A transmission electron microscopic study of microglia/macrophages in the hippocampal cortex and neocortex following chronic exposure to valproate. *Int J Exp Pathol* 86, 91-96.