

Supplemental Information

Astrocytic Insulin Signaling Couples

Brain Glucose Uptake with Nutrient Availability

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Material & Methods

Genotyping of mouse models

The genotyping of loxP sites was performed by PCR using primer sets specifically designed for IR^{ff} to bind to: oKAHN03: 5- GAT GTG CAC CCC ATG TCT G-3'; oKAHN04: 5-TCT ATC AAC CGT GCC TAG AG-3'; oKAHN05: 5'-CTG AAT AGC TGA GAC CAC AG-3', for UCP2^{ff} to bind to: UCP2 forward: 5'-ATC TGA GCA CCA GGG CTG TC-3'; Ucp2 reverse: 5'-GCA TTT GGA AGG CTG AGG-3' or for LepR^{ff} to bind to: LepR 65A: 5'-AGA ATG AAA AAG TTG TTT TGG GA-3'; LepR 105: 5'-ACA GGC TTG AGA ACA TGA ACA C-3'; LepR 106: 5'-GTC TGA TTT GAT AGA TGG TCT T-3'.

Genotyping of hGFAP-CreER^{T2} was performed by PCR using primer sets binding to Cre (Cre-1084, 5'-GCG GTC TGG CAG TAA AAA CTA TC-3'; Cre-1085, 5'-GTG AAA CAG CAT TGC TGT CAC TT-3'; Cre-42, 5'-CTA GGC CAC AGA ATT GAA AGA TCT-3'; Cre-43, 5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3').

Genotyping of GLAST^{CreERT2} was performed by PCR using primer sets binding to Cre (Forward GLAST F8: 5'-GAG GCA CTT GGC TAG GCT CTG AGG A-3'; Reverse GLAST R3: 5'-GAG GAG ATC CTG ACC GAT CAG TTG G-3'; CreERT2 specific primer: 5'-GGT GTA CGG TCA GTA AAT TGG ACA T-3').

Genotyping of Rosa26 ACTB-tdTomato/EGFP was performed by PCR using primer sets binding to 7318: 5'-CTC TGC TGC CTC CTG GCT TCT-3'; 7319: 5'- CGA GGC GGA TCA CAA GCA ATA-3'; 7320: 5'-TCA ATG GGC GGG GGT CGT T-3'. Genotyping of hGFAP-eGFP was performed by PCR using primer sets binding to GFAP-LZ1: 5'- ACT CCT TCA TAA AGC CCT CG-3'; GFP-2: 5'- AAG TCG ATG CCC TTC AGC TC-3'.

Feeding Experiments

For fasting-refeeding experiments, the bedding material was changed and mice were fasted overnight. At about 0900 h, food was added and its consumption was measured for the following 6 h.

To assess glucose-suppression of feeding behavior, glucose was dissolved in Phosphate-buffered saline (PBS) and injected *ip* (2 g/kg bw) or into the lateral cerebral ventricle (1 mg in 2 μ l) after overnight food deprivation. At about 0900 h, food was provided 30 min after injection and, its consumption was measured for the subsequent 4 h.

For the glucodeprivation experiments, 2DG was dissolved in PBS and injected *ip* (10 μ l/g at 250 or 500 mg/kg bw) or into the lateral ventricle (1 mg in 2 μ l) at 1200 h, 3 h after food removal. Food was provided 30 min after the injection and intake was measured for the following 3-4 h as the loss of food weight.

Cerebrospinal Fluid collection

The dura above the cisterna magna was exposed through careful microdissection. Cerebrospinal fluid (CSF) was collected from the cisterna magna using a pulled glass micro-capillary and was microscopically inspected for blood contamination (contaminated samples were discarded). CSF samples were collected after 30 min of *ip* injection of vehicle (PBS) or glucose (2 g/kg bw) and immediately frozen and kept at -80°C until further analysis.

¹⁸F-FDG PET imaging

[¹⁸F] fluorodeoxyglucose (¹⁸F-FDG) and positron emission tomography (PET) were used to measure brain glucose handling. Briefly, fasted mice were injected *ip* with the positron emitting radiotracer ¹⁸F-FDG (18.5 MBq in 0.2 ml of 0.9% NaCl, Instituto Tecnológico PET, Spain). During an uptake period of 45 min, animals were anesthetized by inhalation of a mixture of isoflurane/oxygen (5% for induction and 2% for maintenance) and then placed on the bed of the tomograph. The duration of the PET acquisition was 20 min, immediately followed by a CT (computed tomography) scan. The scanner used was a specific small animal PET-CT hybrid tomograph (Albira ARS, Oncovision, Spain). After acquisition, PET images were reconstructed with an ordered, subset-expectation maximization (OSEM) algorithm and with applied corrections for randomness, scatter, attenuation, dead time and radio-element decay, whereas for the CT images a filtered back projection algorithm was used. For metabolic activity quantification, the procedure used was as follows: first, the CT image of the skull from each animal was co-registered to a magnetic resonance image (MRI) template of mouse brain in which the regions of interest (ROIs) were previously delineated. After the CT image was co-registered, the spatial mathematic transformation was saved and then applied to its own, fused PET image, allowing the correct matching between the PET image and the MRI template. Once the ¹⁸F-FDG uptake in the different brain regions was calculated (in kBq/cc units), the activity of each left hemisphere region was normalized to its homologous region in the right hemisphere and expressed as proportional uptake (left/right). All processes of visualization, co-registration and quantification were performed using PMOD 3.0 software (PMOD Technologies Ltd., Switzerland).

Intracerebroventricular (icv) injections

Twelve-weeks old hypothalamic GFAP-IR WT and KO littermates mice were anesthetized with a mixture of ketamine and xylazine (100 mg/kg and 7 mg/kg, respectively), and implanted with stainless steel cannulas (Bilaney Consultants GmbH, Düsseldorf, Germany) into the lateral cerebral ventricle. Stereotaxic coordinates were -0.8 mm posterior and -1.4 mm lateral from bregma and -2.0 mm ventral from the dura. Correct placement of the cannula was verified by *icv* administration of angiotensin II (1 µg/µl of 0.9% saline). Mice that failed to drink water within 30 min were removed.

Fluorescence-activated cell sorting (FACS)

We isolated GFP-targeted hGFAP positive cells from the brain of IR^{fl/fl} or hGFAPCreER^{T2} mice crossed with hGFAP-eGFP reporter mice (Nolte et al., 2001). The collection of GFP-expressing cells was performed 6 weeks after Tx or vehicle injection. Cells were prepared for FACS sorting as previously described (Beckervordersandforth et al., 2010). Fluorescent cells were sorted using a FACS Aria III (BD Biosciences, Heidelberg, Germany) in the single-cell mode at the appropriate sort rate. A negative control of non-fluorescence cells was used to determine that the background fluorescence was less than 1% of non-fluorescent cells and was included in the sort gate. Sorted cells were collected in RTL buffer supplemented with 1% of β-mercaptoethanol and stored at -80°C until RNA extraction.

Blood and Cerebrospinal fluid analysis

Blood was collected in centrifuge tubes and centrifuged at 4°C and 2000 xg for 15 min to generate serum and stored at -80°C. Insulin levels were measured by an ultrasensitive murine insulin ELISA kit (Crystal Chem, Inc., Downers Grove, IL, USA). Glucose levels were measured by a Glucose Assay Kit (Abcam, ab65333, Cambridge, UK). All assays were performed according to the manufacturers' instructions.

In situ Hybridization (ISH) combined with immunocytochemistry

For *in-situ* hybridization, we injected Tx to IR^{ff} and GLAST^{CreERT2}:IR^{ff} mice crossed with tdTomato/eGFP mice and injected with Tx (GLAST-IR WT and GLAST-IR KO mice, respectively). Two wk after Tx injection, mice were perfused with 4% Paraformaldehyde (PFA) and brains were collected in RNase free conditions and sectioned (30 µm) on a cryostat. Sections were treated with 0.1 M PBS-buffered methanol and H₂O₂ for 10 min, 0.2 N HCl for 10 min, proteinase K for 30 min at 37°C, 0.2% glycine buffer 30 s and 0.1% Triton X-100 for 10 min. Brain sections were hybridized in hybridization buffer (Sigma-Aldrich) containing 200 nM locked nucleic acid (LNA) – modified cDNA probes labelled with digoxigenin (Exiqon, Denmark) at 54°C overnight. Scrambled DNA probe was included as a negative control (5'DIG-GTGTAACACGTCTATACGCCCA-3'DIG). The probe was designed according to exon 4 of the mouse insulin receptor (NM_010568.2). After stringent washing in 2X, 0.5X and 0.2X SSC buffer at 54 °C and final washing in 0.1 M PBS, sections were incubated with goat anti-digoxigenin antibody (1:200, Abcam) and rabbit anti-GFP (1:400, abcam) at 4 °C overnight. Sections were incubated with biotinylated anti-goat secondary antibody for 1 h and then

incubated with streptavidin conjugated with Alexa 647 (1:500, for Digoxigenin) and donkey anti-rabbit antibody conjugated by Alexa 488 (1:400, for GFP) (all from Jackson ImmunoResearch Laboratories). After thorough rinsing, sections were mounted with mounting medium and visualized by confocal microscopy (Zeiss-LSM710, Germany).

Immunofluorescence

Mice were euthanized in CO₂ and perfused with saline, followed by paraformaldehyde 4% in PBS 0.1 M (pH=7.4) cooled to 4°C. Brains were dissected and post-fixed for 24 h at 4°C. After post-fixation, brains were transferred for 48 h to a 30% sucrose solution in 0.1 M Tris-buffered saline (TBS; pH 7.2) and sectioned (30 µm) on a cryostat. Sections were incubated overnight with primary antibody at 4°C. Sections were then washed 3 times and incubated for 1 h with secondary antibodies. A solution containing gelatin (0.25%) and TritonX100 (0.5%) in TBS was used for antibody incubations. The following primary antibodies were used: Phosphorylated protein kinase B (pAkt, rabbit, 1:100, Cell Signaling, Danvers, MA, USA), rabbit anti-POMC precursor (1:1000, Phoenix Pharmaceuticals Inc. CA, USA), rabbit anti-c-Fos (1:500, Santa Cruz Biotechnology, Inc, TX, USA), goat anti-GFP (1:1000, Abcam), mouse anti-S100β (1:1000, Abcam, Cambridge, UK), goat anti-GFAP (1:1000, DAKO Deutschland GmbH, Hamburg, Germany) and mouse anti-GFAP (Sigma, 1:1000) diluted in SUMI (gelatin 0.25% and TritonX100 0.5% in TBS) at 4°C. After several TBS washes, sections were incubated with the following secondary antibodies: for pAkt, anti-rabbit biotinylated 1:500 and streptavidin (STV) conjugated with Alexa fluor 488 antibody (Vector Biolab).

For pIRS1, anti-rabbit alexa fluor 657 was used (1:1000, Molecular Probes, Life Technologies GmbH, Darmstadt, Germany). For S100 β and GFAP, anti-mouse or anti-goat alexa fluor 647 or 555 was used (1:2000, Molecular Probes). For c-Fos, alexa fluor 555 was used (1:1000 Vector Biolab).

Visualization and Quantification of Cre expression in astrocytes

For the evaluation of cellular distribution of hGFAP and GLAST in the brain, we analyzed the localization of Cre by immunofluorescence in C57BL6J WT mice (for the viral-based model) or GLAST^{CreERT2} mice (tamoxifen-conditional model) crossed with tdTomato/eGFP mice. (Jackson Laboratories, Stock No. 007576), which allow the visualization of GFP expression in cells undergoing Cre recombination after Tx injection, occurring under the control of the hGFAP or GLAST promoter, respectively.

Colocalization images were captured by a confocal microscopy (Zeiss-LSM710) at 20X magnification. Next, we quantified the specificity of Cre-recombination as the number of S100 β or GFAP that overlapped with GFP-expressing cells (Cre-recombined cells), and the efficiency of Cre-recombination as number of GFP-expressing cells that showed immunoreactivity for S100 β and GFAP in both models. For the tamoxifen-conditional mouse model, we analyzed several fields corresponding to a field of 450x450 μm^2 . For the viral-based model, we analyzed several fields corresponding to an area of 200x200 μm^2 . Specificity was determined by calculating the ratio of eGFP+ cells/ S100 β + or GFAP+ cells; whereas efficiency was determined by calculating the ratio of S100 β + or GFAP+ cells/ eGFP+ cells.

Immunocytochemistry for Electron Microscopy

Mice were deeply anesthetized and the left heart ventricle was rapidly cannulated and flushed with 0.9% saline followed by freshly prepared fixative (paraformaldehyde 4%, glutaraldehyde 0.1%, in PBS 0.1 M, pH=7.4). Brains were dissected and post-fixed overnight in fixative without glutaraldehyde. After washing in cold phosphate buffer (0.1M), vibratome sections (50 μ m) containing the hypothalamic brain nuclei were cut. Sections were washed in PBS several times, cryo-protected and subsequently frozen and thawed 3 times in liquid nitrogen. After extensive washing in PBS, slices were incubated with H₂O₂ (1%, 20 min, room temperature, shaking) to block endogenous peroxidase activity. After washing again with PBS, sections were incubated with primary antibody anti-POMC (1:4000, Phoenix Pharmaceutical Inc, CA, USA) at 4°C for 48 h with gentle shaking. Sections were extensively washed, incubated with secondary antibody (2 h, room temperature), washed again, put in avidin-biotin conjugates (ABC) and developed with DAB. Sections were then osmicated (15 min in 1% osmium tetroxide in PBS) and dehydrated in increasing ethanol concentrations. During the dehydration, 1% uranyl acetate was added to the 70% ethanol to enhance ultrastructural membrane contrast. Dehydration was followed by flat embedding in Durcupan. Ultrathin sections were cut on a Leica ultra microtome, collected on Formvar-coated single-slot grids, and analyzed with a Tecnai 12 Biotwin (FEI) electron microscope. All investigators were blind regarding the experimental groups during the entire procedure.

Quantification of Astrocyte Number and Morphology

The analyses of the number of GFAP immunoreactive (GFAP+) cells and their morphology were performed using ImageJ software. Briefly, 4 sections/mouse (4 mice per group) distributed from -1.6 mm to -1.9 mm from Bregma were analyzed. Images of a single focal plane of hypothalami (containing the MBH) and hippocampi (containing the CA1, CA2, CA3 regions) in a corresponding area of 18.5 mm² were captured at 10 X magnification by a BZ-9000 microscope. The number of GFAP+ (cells per field), primary projection and mean projection length were determined using Image J, as previously described (Fuente-Martin et al., 2012).

All morphometric analyses were performed in blinded fashion (without previous knowledge of the identity of the experimental groups).

Quantification of pAkt in GFAP positive cells in the Mediobasal Hypothalamus

To validate genetic or viral-mediated deletion of IRs in GFAP+ cells, we quantified the number of GFAP + cells that showed immunoreactivity to pAkt 15 min after vehicle (PBS) or insulin injection (*ip*; 3 U insulin/kg BW). Twenty fields (10 x 12 mm²) per group (4 mice per group) were analyzed at 10 X magnification. MBH slices (-1.6 mm to -1.9 mm from Bregma) were imaged by using BZ-9000 microscope.

Quantification of c-Fos immunoreactivity in specific hypothalamic brain areas

c-Fos immunoreactive (cFos+) cells were quantified in the brain of GFAP-IR WT or KO mice 2 h after vehicle (PBS) or glucose injection (2g/kg BW) by using ImageJ. Images were captured at 10 X magnification by a BZ-9000 microscope (Keyence). The number of c-Fos positive neurons was calculated in 5-10 sections/mouse for the analysis of different hypothalamic nuclei (ARC, DMN, VMN and LH). The analysis was performed according to the Allen Mouse Brain Atlas (position -1.6 mm to -1.9 mm from Bregma). Quantification of c-Fos immunoreactivity in POMC neurons was performed by counting the percentage of c-Fos and POMC-immunolabelled cells. For each mouse, 15-20 sections containing the ARC were analyzed. All analyses were performed in blinded fashion (without previous knowledge of the identity of the experimental groups).

Quantification of Astrocytic Coverage and Synaptology on POMC neurons

The analysis of the astrocytic coverage of the cell membrane of labeled POMC cells was assessed by using both light and electron microscopy. For light microscopic analysis, brain sections were labeled with GFAP and POMC-precursor protein (for detailed information see above). Single plane images were captured by confocal microscopy (Zeiss-LSM710) using a 63 x objective (digital zoom x3). For quantification, the total perimeter of POMC-immunoreactive perikarya was assessed (100%), as were the appositions between POMC-neurons and GFAP-immunoreactive processes by using Image J. For electron microscopy, microscopic photographs (11,500X) were used to first measure the perimeter of each POMC cell analyzed, followed by

determination of the amount of membrane covered by astrocytes (in nm). We used the same images to also analyze the total number and type of synapses (symmetric and asymmetric) along the perimeter of POMC cells. The results are reported as astrocyte coverage or number of total, symmetric and asymmetric /cellular perimeter of the POMC cell.

Mitochondria and Mitochondria-ER quantification

Hypothalamic sections containing GFAP or POMC immunoreactive cells with a visible nucleus were analyzed by electron microscopy. A blind investigator manually traced mitochondrial profiles using ImageJ. All cells were checked twice for consistency. Mitochondrial cross-sectional area and the mitochondria aspect ratio (AR, the major axis divided by the minor axis, minimum value is 1.0) were calculated. AR was utilized as a shape descriptor. Probability plots were utilized to estimate changes in mitochondrial size and shape and statistical differences were tested using the Kolmogorov-Smirnov test. Mitochondrial density was estimated by dividing the number of mitochondrial profiles by the cytosolic or cellular areas. Mitochondrial coverage was estimated by dividing the total area of mitochondria by the cytosolic or cellular areas. Differences in mitochondrial density and coverage were tested using t-test. For the mitochondria-ER interaction, a blind investigator scored the number of mitochondria in contact with ER profiles in high magnification images (> 11,000 x). $P \leq 0.05$ was considered statistically significant.

Laser-Capture Micro Dissection and Real-time PCR

To validate that the viral-based model is a suitable Cre/loxP approach to specifically ablate astrocytic IRs, we used adult WT (C57BL6J background) mice or IR^{ff} mice crossed with tdTomato/eGFP reporter mice. Two weeks after bilateral virus injection, mice were anesthetized by CO₂, and perfused with saline and 4% PFA. Brains were collected and post-fixed in 4% PFA at 4 °C for 24 h, and subsequently transferred to RNase-free sucrose solution (30% sucrose in 0.1 M Tris-buffered saline, pH 7.2) for 24 h. Brain coronal sections (30 μm) were cut by a cryostat and transferred to PEN membrane frame slides (Life technologies, LCM0521). A laser-capture microdissection system (Leica, LMD7000) was used to dissect the area of viral infection by the means of a fluorescent camera, as depicted in Fig. S3F.

Fifteen to twenty hypothalamic sections per mouse were dissected. RNA was isolated by an Arcturus Paradise RNA isolation kit (Life technologies, KIT03121) in a total elution volume of 15 μl. RNA quality was tested by the means of a Bionalyzer (Agilent 2100, Agilent, Santa Clara, CA, USA). For each mouse, 200 ng of total RNA was used for cDNA reverse transcription (QuantiTech reverse transcription kit, Qiagen). Quantitative PCR analysis was performed by using TaqMan Gene Expression Assays and the TaqMan FAST Universal PCR Master Mix (Applied Biosystems). For each reaction, 2 μl of a 1:2 dilution of the RT product was used in a final volume of 10 μl, and each sample was assayed in duplicate.

Energetic pathway studies

Primary hypothalamic astrocytes were cultured in XF24 plates (Seahorse Bioscience) at a concentration of 200,000 cells/well and then maintained for 3

h with DMEM medium without glucose. Next, the XF24 plates were washed with XF assay medium and incubated for 1 h with or without etomoxir (40 μ M; Sigma) in a 37°C non-CO₂ incubator. After incubation, the XF24 plates were transferred to a temperature-controlled (37°C) XF24 extracellular flux analyzer (Seahorse Bioscience) and subjected to an equilibration period. One measurement cycle was comprised of 1 min mixing, 2 min waiting and a 3-min measuring period. After basal cycles, oligomycin (1 μ g/ml) was added by automatic pneumatic injection to inhibit the ATP synthase and thus approximate the proportion of respiration used to drive ATP synthesis and mitochondrial proton leak. After further assay cycles, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 0.5 μ M) was injected to stimulate maximal respiration in mitochondria by removing respiratory control. Rotenone (4 μ M) and antimycin (2 μ M) were added with the last injection to determine the non-mitochondrial respiration rate, which was subtracted from all other respiration rates for analysis. The coupling efficiency was calculated as the oligomycin-sensitive fraction of mitochondrial respiratory activity. To determine extracellular acidification rates (ECARs) for estimation of glycolysis, the measurements were ended by addition of 2DG (100 mM). ECARs were converted into proton production rates (PPRs) by taking into account the buffer capacity of the medium.

Glucose Uptake Colorimetric Assay

Glucose uptake was measured from primary cultures according to the manufacturer's instructions (ABCAM, ab136955). The cells were incubated for 6 h with DMEM without glucose (Life Technologies) and then incubated with

2-DG, which was taken up and metabolized to 2-DG-6-phosphate (2-DG6P). The accumulated 2-DG6P was directly proportional to 2-DG (or glucose) uptake by cells. The 2-DG6P was oxidized to generate NADPH, which was determined by an enzymatic recycling amplification reaction.

Glycogen Quantification and L-Lactate Assay

Primary hypothalamic astrocytes were incubated for 6 h with DMEM without glucose (Life Technologies) and then were exposed to glucose (5 mM) overnight on the next day, the supernatants were collected and stored at -80°C for L-Lactate determination, and cells were washed and lysated in ice-cold water and stored at -80°C until glycogen determination.

For glycogen quantification, a small fraction (~5 µL) of cleared cell lysates was taken for protein quantification, and the remainder was boiled (100 °C) for 5 min to inactivate enzymes. Soluble glycogen was collected in the supernatant after subsequent centrifugation at 13000 × *g* for 5 min. Twenty-five µl of the glycogen sample were used for glycogen quantification using a Glycogen Assay Kit (Sigma, MAK016-1KT) and based on a coupled-enzyme assay, which produced a colorimetric (570 nm) product proportional to the glycogen present. Glycogen concentration was normalized to 1 µg of total protein.

For L-lactate quantification, a BioAssay Systems' EnzyChrom™ lactate assay kit (Bioassays Systems, Hayward, CA, USA) was used for colorimetric determination of L-Lactate from media samples of astrocyte cultures. The assay was based on lactate dehydrogenase-catalyzed oxidation of lactate, in which the formed NADH reduced a formazan (MTT) reagent. The intensity of

color product was measured at 565 nm, which was proportional to the lactate concentration in the sample.

Protein extraction and Western Blotting

Protein content from primary cells and tissues was extracted using RIPA buffer containing protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc., Rockford, IL USA), 1 mM phenyl-methane-sulfonyl fluoride (PMSF) and 1 mM sodium butyrate (Sigma). Proteins were transferred on nitrocellulose membranes using a Trans Blot Turbo transfer apparatus (Biorad, Hercules, CA, USA) and stained with IR β (Santa Cruz, 1:500), anti-pAkt in Ser473 (rabbit polyclonal, 1:500), total Akt (rabbit polyclonal, 1:2000) both from Cell Signaling Technology and GAPDH (mouse polyclonal, 1:5000; Santa Cruz). Detection was carried out on a LiCor Odyssey instrument (Lincoln, NE, USA), using ECL (Biorad).

RNA isolation and qPCR analysis

RNA was isolated from tissues and primary cultures using a commercially available kit (Micro RNeasy kit, Qiagen, Hilden, Germany). For qPCR analyses, equal amounts of RNA were transcribed to cDNA using Superscript III (Invitrogen, Darmstadt, Germany). Gene expression was analyzed using taqman probes (Applied Biosystems, Carlsbad, CA, USA) and PCRs were carried out using a ViiA™ 7 Real Time PCR System (Applied Biosystems). Gene expression was evaluated using the $\Delta\text{-}\Delta$ Ct method and 18S, TBP or HPRT was used as a house keeping gene. Taqman probes used were IR (Mm01211875_m1) and CPT1C (Mm00463970_m1) for brain-purified

astrocytes and hypothalamic astrocyte cultures, and GLUT-1 (Mm00441480_m1) from brain tissue and cortical astrocyte cultures. All Taqman probes were provided by Applied Biosystem.

Supplemental References

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