APPENDIX

Table of contents:

1. Appendix Supplementary Methods

2. Appendix Supplementary References

3. Appendix Figures

1. Appendix Supplementary Methods

Material and methods

Cell culture and stimulation

Cortical neurons from E21 Sprague Dawley rats were cultured as described previously (Martorell-Riera et al, 2015). Experiments were performed after a culturing period of 10–11 days during which cortical neurons develop a rich network of processes, express functional NMDA-type and AMPA/kainate-type glutamate receptors, and form synaptic contacts. Prior to stimulations and transfections, neurons were transferred from growth medium to a medium composed of 10% MEM (Invitrogen) and, 90% salt-glucose-glycine (SGG) medium (SGG: 114 mM NaCl, 0.219 % $NaHCO_3$, 5.292 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 1 mM glycine, 30 mM glucose, 1 mM glutamine, 0.5 mM sodium pyruvate, 0.1% phenol red; osmolarity 325 mosm/l). Bursts of action potential firing were induced by treating of neurons with 50 µM bicuculline (Sigma), and burst frequency was enhanced by adding of 250 µM 4-amino pyridine (Sigma).

HIF-1α gene inactivation in vivo

To study the in vivo role of HIF-1α in neuronal architecture, we used previously described HIF1α floxed UBC-CRE-ERT2 mice (Soro-Arnaiz et al, 2016). These mice ubiquitously express a tamoxifen-inducible CRE recombinase (cre-ERT2) that allows global inactivation of HIF-1α locus flanked by two LoxP sites upon 4OH-tamoxifen treatment. For HIF-1α gene inactivation, the newborn mice received tamoxifen via breast feeding from the mother. Weaning mothers were injected intraperitoneally with 4OH-tamoxifen daily for 5 days (2 mg/day) starting 3-5 days postpartum. After this period females were returned to a standard mouse diet. Once newborns reached adulthood (4-6 months) neuronal architecture was analyzed. Mice were kept under specific pathogen-free conditions at the animal facility at the Autonomous University of Madrid (UAM).

3PO administration

P8 rat littermates were daily injected intraperitoneally for 5 days with 50 mg/kg PFKFB3 inhibitor 3PO (Merck) or DMSO (control). Six hours after the last injection, pups were sacrificed and brain samples were extracted to perform Golgi staining. Rats were kept under specific pathogen-free conditions at the animal facility at the Barcelona University (UB).

Histology

PFA-fixed brain mice (10-days and 3-months old) coronary sections (30 μm thicknes) were blocked for 2 hours at room temperature with PBS containing 10% of normal goat serum (NGS), 0.2% of gelatin. Anti-HIF-1α antibody (1:250) was incubated overnight at 4 °C with PBS-5% NGS. Sequential incubation with biotinylated anti-rabbit secondary antibody for 2 hours at room temperature (1:200; 711-065-152. Jackson Immunoresearch) followed with streptavidin-HRP incubation for 2 hours at room temperature temperature (1:400; Vectastain ABC kit, Vector Laboratories) was performed in PBS–5% NGS. Bound antibodies were visualized by

reaction using DAB and H_2O_2 as peroxidase substrates (Vectastain ABC kit, Vector Laboratories). Finally, sections were dehydrated and mounted.

For antigen immunodetection, sections were blocked for 2 h at RT with PBS-0.1% Triton-X-100 (PBST) containing 10% of fetal bovine serum (FBS), 0.2% of gelatin, and F(ab')2 fragment anti-mouse IgG (Jackson ImmunoResearch,1:300) when needed. Primary antibodies were incubated overnight at 4°C with PBS–5% FBS.

Anti-NeuN (Millipore) and anti-GFAP (DAKO) antibodies, dye-labeled secondary antibodies (1:300) were incubated for 2 h at RT in PBST–5% FBS. For the immunohistofluorescent detection of HIF1-α (Abcam), sequential incubation with biotinylated secondary antibodies in PBST–5% FBS (1:300; 2 h at 4ºC) and streptavidin-HRP (VECTASTAIN® Elite® ABC-HRP Kit, Vector Laboratories) (2 h at 4ºC) was performed . Bound antibodies were visualized by reaction using TSA® Plus Cyanine 3 detection kit (Perkin Elmer). Finally, sections were counterstained with DAPI (Sigma, 5 µm), mounted in Fluoromont and stored at −4°C.

For Golgi staining, impregnation was performed using Rapid Golgi Stain Kit (FD Neurotechnologies) following the manufacturer's protocol. Brains were stored in impregnation solution for two weeks in the dark. Coronal brain sections (150 µm thick) were obtained by slicing frozen brains with a cryostat, mounted in gelatin-coated slides and allowed to dry naturally at room temperature. Sections were then incubated during 10 minutes with staining solution, dehydrated and mounted with Eukitt (Sigma). Layer 5 pyramidal neurons of the somatosensory cortex were analyzed.

Transfection, plasmids and virus generation

Neurons were transfected at DIV8 using Lipofectamine 2000 (Invitrogen). Transfection efficiency was approximately 5 % in which nearly the totality of transfected cells are neurons (Soriano et al, 2008). For knockdown experiments 25 ng of rat Glut3 (D-090091-01), rat ACLY

(M-098529-01-0005), rat Siah2 (L-089773-02) or non-targeting control (D-001810-10-05) siRNA-SMART pool (Thermo scientific) containing a pool of 4 siRNAs were used.

HRE-luciferase was a gift from Navdeep Chandel (Emerling et al, 2008) (Addgene plasmid # 26731); pCDNA3-HA-HIF-1α a gift from H Frankling Bunn (Huang et al, 1998); ODD-Luciferase-pcDNA3 (Addgene plasmid # 18965) and Luciferase-pcDNA3 (Addgene plasmid # 18964) were gifts from William Kaelin (Safran et al, 2006); CRE-luc and ICER were gifts from Giles Hardingham (Papadia et al, 2005).

The vectors used to construct and package recombinant adeno-associated viruses (rAAVs), pAAV-sh-sc, pAAV-GFP and pAAV-A-CREB were provided by Hilma Bading (Zhang et al, 2007). To construct pAAV-HIF-DN, the first 1,020 bp of the HIF-1α cDNA (from pCDNA3-HA-HIF-1α) were amplified using the following primers: forward 5'- AGA *GGA TCC* TAC CCA TAC GAT GTT CCA GAT -3' and reverse 5'- AAG CG*G ATA TC*T AAT TCA CAC ATA CAA TGC ACTG -3'. The amplified product contains sequences with BamHI and EcoRV restriction sites at the 5' and 3' respectively (italics). GFP in the rAAV-GFP vector was removed by BamHI/EcoRV digestion and the HIF-DN PCR product was cloned into the rAAV vector to express the first 340 amino acids of the HIF-1α N-terminus, which contains the DNA binding and dimerization domains, but not the ODD and transactivation domains. rAAV for shRNA expression contains the U6 promoter for shRNA expression and a CMV/chicken beta-actin hybrid promoter driving hrGFP expression. rAAV-shRNA was made by swapping the sh-sc sequence of rAAV-sh-sc for the following sequences of the rat gene into the BamHI and HindIII restriction sites: shSiah2(1): 5'- ACA GAG AAA CCA GAG CAT GAA -3'; shSiah2(2): 5'- GCA AGC AAG CAG AGA ACT TTG-3'; shGPI: 5'- GGA TTA CTC CAA GAA CCT TGT-3'; shACLY: 5'- GCA TCA AGC CTG GAT GCT TTA -3'. All newly generated constructs were confirmed by sequencing.

Neurons were infected with rAAV at DIV4. Infection efficiencies were determined at DIV 10-11 by analyzing GFP fluorescence or immunocytochemical analysis and were observed to range from 70 to 85% of the viable neurons.

Neurite length measurement

Cortical neurons were transfected with a plasmid expressing GFP and neurons were fixed 48 hours later with 4% paraformaldehyde, permeabilized, blocked and incubated over-night at 4°C with anti-GFP antibody (1:750, A11122, Life Technologies). Antibody binding was visualized using a biotinylated secondary antibody (1:200, Jackson Immuno Research) and Cy3-conjugated streptavidin (1:500, Jackson Immuno Research). Preparations were mounted on VECTASHIELD Mounting Medium with DAPI (Vector Laboratories).

Images were taken blindly at 4X magnification using an Olympus BX61 microscope equipped with an Olympus DP70 camera. Neurites were manually traced and analyzed using Simple Neurite Tracer software (Longair et al, 2011).

Luciferase assays

Cells were transfected with firefly luciferase-based reporter plasmid along with a Renilla expressing vector (pTK-RL; Promega), together with, where relevant, an HIF-DN or A-CREB expression vector. Luciferase assays were performed using the Dual Glo Luciferase Assay system (Promega) with firefly luciferase-based reporter gene activity normalized to the Renilla control (pTK-RL plasmid), except the CMV-ODD-Luc and CMV-Luc experiments that were normalized to CMV-Renilla.

RNA isolation, RT-PCR and qPCR

RNA was isolated using an PureLink™ RNA mini kit (Life Technologies). For qPCR, cDNA was synthesized from RNA using the SuperScript® III First-Strand Synthesis SuperMix (Life

Technologies) following the manufacturer's instructions. qPCR was performed in a StepOne Real-Time PCR System (Applied Biosystem) using GoTaq qPCR Master Mix (Promega) according to the manufacturer's instructions. The primers used were:

Glut3 -F: 5'- CAT CTC CGT TGT CCT CCA GT -3', -R: 5'- GCT CCA ATC GTG GCA TAG AT -3'; HK2 -F: 5'- CCA GCA GAA CAG CCT AGA CC -3, -R: 5'- AGA TGC CTT GAA TCC CTT TG -3'; PFK –F: 5'- CTG GGA GAG CGT GTC CAT-3', -R: 5'- CAT CGG GCA CTT CCA ATC -3'; PFKFB3 –F: 5'- ACA ATG AGG AGG CCA TGA GA -3', -R: 5'- CTT TGT CAG GTA GCT TTT GAC G -3'; PKM –F: 5'- GCC GCC TGG ACA TTG ACT C -3', -R: 5'- CCA TGA GAG AAA TTC AGC CGA G -3'; Siah2 –F: 5'- ATG CCG CCA GAA GTT GAG -3', -R: 5'- GTA TGG TGT AGA GTC AGG GAA CAG -3'; HIF-1α –F: 5'- AAC AGG ATG GAA TGG AGC AG -3', -R: 5'- TGG TCA GCT GTG GTA ATC CA -3'; GPI –F: 5'- AGT ACA TGC ACC GCT TTG CT-3', -R: 5'-ACT TTC CAT TGG ATT CCA TGT C-3'; Siah1 –F: 5'- TCTCCGCCCACAGAGATGAG -3', -R: 5'- GTTGGATGCAGTTGTGCCAG -3'; 18S -F: 5'-GTG GAG CGA TTT GTC TGG TT-3', -R: 5'-CAA GCT TAT GAC CCG CAC TT-3'. Expression of the gene of interest was normalized to that of 18S rRNA, a commonly used control.

Western blotting and antibodies

Mice cortices were frozen with liquid nitrogen and homogenized by pipetting and passing through a 29G syringe in a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl 1mM EDTA, 1% Triton X-100, 1:00 Protease Inhibitor Cocktail Set III (EMD Milipore), 1:200 Phosphatase Inhibitor Cocktail II and III (Sigma) and 10 µM MG-132 (Sigma). Protein levels were quantified using Pierce BCA Protein Assay Kit (Thermo Scientific).

Total cell lysates were boiled at 100°C for 5 minutes in 1.5x sample buffer (1.5 M Tris pH 6.8; 15% Glycerol; 3% SDS; 7.5% β-mercaptoethanol; 0.0375% bromophenol blue). Gel electrophoresis was performed using 9% polyacrylamide gels. The gels were blotted onto PVDF membranes, that were then blocked for 1 hour at room temperature with 5% (w/v) non-fat dried milk in PBS with 0.05% Tween 20. The membranes were then incubated overnight at 4°C

with the primary antibodies diluted in blocking solution as follows: anti- Glut3 (1:750; ab191071, Abcam), PFKFB3 (1:1000, #13123, Cell Signaling Technology), PKM (1:750; #3190, Cell Signaling Technology), HK2 (1:500; sc-6521, Santa Cruz Biotechnology), HIF-1α (1:2000; ab179483, Abcam), Siah2 (1:350; sc-5507, Santa Cruz Biotechnology), Actin (1:10000, A4700, Sigma), FASN (1:500; sc-48357, Santa Cruz Biotechnology), ACLY (1:1000; #4332, Cell Signaling Technology), HIF2α (1:1000; ab179825, Abcam), PHD1 (1:1000; ab113077, Abcam); PHD2 (1:200; sc-271835, Santa Cruz Biotechnology), PHD3 (1:1000; NB100-139SS, Novus Biologicals), FIH-1 (1:500; sc-271780, Santa Cruz Biotechnology), OGDH (1:500; HPA020347, Sigma) and DLST (1:250; HPA003010, Sigma). To visualize western blots, HRP-based secondary antibodies were used followed by chemiluminescent detection on Kodak X-Omat film.

Acetyl-CoA and CoA determination

Acetyl-CoA and CoA levels were measured using the Acetyl-Coenzyme A and CoA Assay Kits, respectively (Sigma). Briefly, six well plates were used per condition (around 8 million neurons). Neurons were washed twice with PBS and scraped in PBS containing protease inhibitors (Inhibitor Cocktail Set III, EMD Millipore) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Set 1 and 3, Sigma). Neurons were pelleted, resuspended in 240 μl of lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and protease and phosphatase inhibitors) and sonicated using a Diagenode Bioruptor (Liege, Belgium; 30 seconds on at full power and 30 seconds off, in an ice bath for 5 minutes). After centrifugation the supernatant was deproteinized using 10K Amicon-Ultra-0.5 mL centrifugal filters (EMD Millipore). Reaction mixtures in triplicate were set up according the kit's instructions.

To determine cytoplasmic acetyl-CoA levels, pelleted neurons were resuspended in ice-cold buffer containing 250 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA, and protease and phospatase inhibitors. Neurons were homogenized using a Dounce homogenizer with a tight

fitting Teflon pestle (15 strokes). The homogenates were centrifuged at 14,000 *g* for 10 minutes and the supernatant deproteinized and assayed following the kit's instructions. Acetyl-CoA levels were normalized by total protein levels, quantified using Pierce BCA Protein Assay Kit (Thermo Scientific).

Glucose uptake measurements

The uptake of 2-[*N*-(7-nitrobenze-2-oxa-1, 3 diazol-4-yl) amino]-2 deoxy-glucose (2-NBDG, Life Technologies), a fluorescent glucose analog, was used to measure glucose transport. Cortical neurons were rinsed 3 times with phenol-red free SGG medium with reduced glucose concentration (0.5 mM) and incubated with 100 μM 2-NBDG in reduced glucose SGG medium for 30 minutes at 37ºC and 5% CO_2. Cultures were washed three times with phenol-red free SGG medium to remove free 2-NBDG. Accumulation of intracellular 2-NBDG, measured using an excitation wavelength of 488 nm, was imaged under a Leica DMIRB microscope equipped with a Leica DFC 550 camera at 40× magnification. ROIs of the same surface were drawn in the soma and fluorescence intensity was analyzed using ImageJ (Schneider et al, 2012).

Imaging studies

Neurons were visualized using a TCS SP2 Leica confocal laser scanning microscope (Leica Lasertechnick GmbH, Mannheim, Germany) adapted to an inverted Leitz DMIRBE microscope at 37°C in a controlled 5% CO2 atmosphere (Life Imaging Services). Pictures were acquired using a 40× (1.25-0.75 NA) Leitz Plan-Apochromatic objective. Images were analyzed using ImageJ software.

Cytoplasmic Ca^{2+} was monitored with Fluo-4 (Life Technologies). Neurons were loaded with 2 μM Fluo-4 for 45 min at room temperature in phenol-red free SGG medium with 10mM HEPES and 10mM glucose. After 3 washes with phenol-red free SGG medium, neurons were de-

esterified for 30 min at room temperature, excited at 488 nm and emission captured with a 516-nm filter.

For hypoxia analysis, neurons were loaded with 10 μM Image-IT Hypoxia Reagent (Life Technologies) in HBSS medium, and placed in an incubator chamber attached to the microscope, which was flushed with 95% $N2$/5% $CO2$ at a flow rate of 20 l/min at 37°C for 30min. Non-hypoxic neurons were maintained in normoxic conditions during probe incubation. Neurons were excited at 490 nm, and emission was measured using a 610-nm filter.

Lactate measurement

The culture medium was filtered using 10K Amicon Ultra-0.5 mL centrifugal filters (EMD Millipore). Then, 50 μL of medium was incubated with 200 μL of reaction buffer (320 mM glycine, 320 mM hydrazine, 2.4 mM NAD^+ and 2 U/mL of lactate dehydrogenase (LDH). After 30 minutes of incubation at room temperature, the lactate-dependent generation of NADH was measured at 340 nm using the Infinite 200 PRO multimode reader (Tecan). Lactate levels were normalized by total protein levels, quantified using Pierce BCA Protein Assay Kit (Thermo Scientific).

Glucose and glutamine incorporation into lipids

Neurons were grown on glass coverslips for 48 hours in a medium containing 0.8 μCi/ml ^{14}C-U-glucose (Perkin-Elmer) or 2 μCi/ml L-3-4-^{3}H(N)-glutamine (Perkin Elmer). Lipid isolation was performed as described previously by Folch et al. (Folch et al, 1957). Briefly, lipids were separated by submerging the coverslips in solution of methanol and chloroform 2:1 (vol:vol), and shaked for 20 minutes at room temperature. After adding one fifth of the volume of 0.154 M NaCl, the samples were centrifuged at 500 x g for 10 minutes at room temperature. 80% of the lower nonpolar phase containing the lipids was added to scintillation liquid and

desintigration per minute was determined by scintillation counting. The values were normalized by protein that was quantified from the polar phase using Pierce BCA Protein Assay Kit (Thermo Scientific).

ATP measurement

ATP levels were measured using the ATPlite Luminescence Assay System (Perkin-Elmer) on the Infinite 200 PRO multimode reader (TECAN) following the manufacteurer's instructions. ATP levels were normalized by total protein levels, quantified using Pierce BCA Protein Assay Kit (Thermo Scientific).

Statistical analysis

Statistical analysis involved two-tailed Student's t-tests. For any multiple comparisons within data sets, we used a one-way ANOVA followed by Tukey's post-hoc test. All data are presented as the mean ± s.e.m. of at least three independent experiments (n). A p value less than 0.05 was considered statistically significant.

2. Appendix Supplementary References

Emerling BM, Weinberg F, Liu J-L, Mak TW, Chandel NS (2008) PTEN regulates p300-dependent hypoxia-inducible factor 1 transcriptional activity through Forkhead transcription factor 3a (FOXO3a). *Proceedings of the National Academy of Sciences* **105:** 2622-2627

Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226:** 497-509

Huang LE, Gu J, Schau M, Bunn HF (1998) Regulation of hypoxia-inducible factor 1α is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proceedings of the National Academy of Sciences* **95:** 7987-7992

Longair MH, Baker DA, Armstrong JD (2011) Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics* **27:** 2453-2454

Martorell-Riera A, Segarra-Mondejar M, Reina M, Martínez-Estrada OM, Soriano FX (2015) Mitochondrial fragmentation in excitotoxicity requires ROCK activation. *Cell Cycle* **14:** 1365-1369

Papadia S, Stevenson P, Hardingham NR, Bading H, Hardingham GE (2005) Nuclear Ca²+ and the cAMP Response Element-Binding Protein Family Mediate a Late Phase of Activity-Dependent Neuroprotection. *The Journal of Neuroscience* **25:** 4279-4287

Safran M, Kim WY, O'Connell F, Flippin L, Günzler V, Horner JW, DePinho RA, Kaelin WG (2006) Mouse model for noninvasive imaging of HIF prolyl hydroxylase activity: Assessment of an oral agent that stimulates erythropoietin production. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 105-110

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Meth* **9:** 671-675

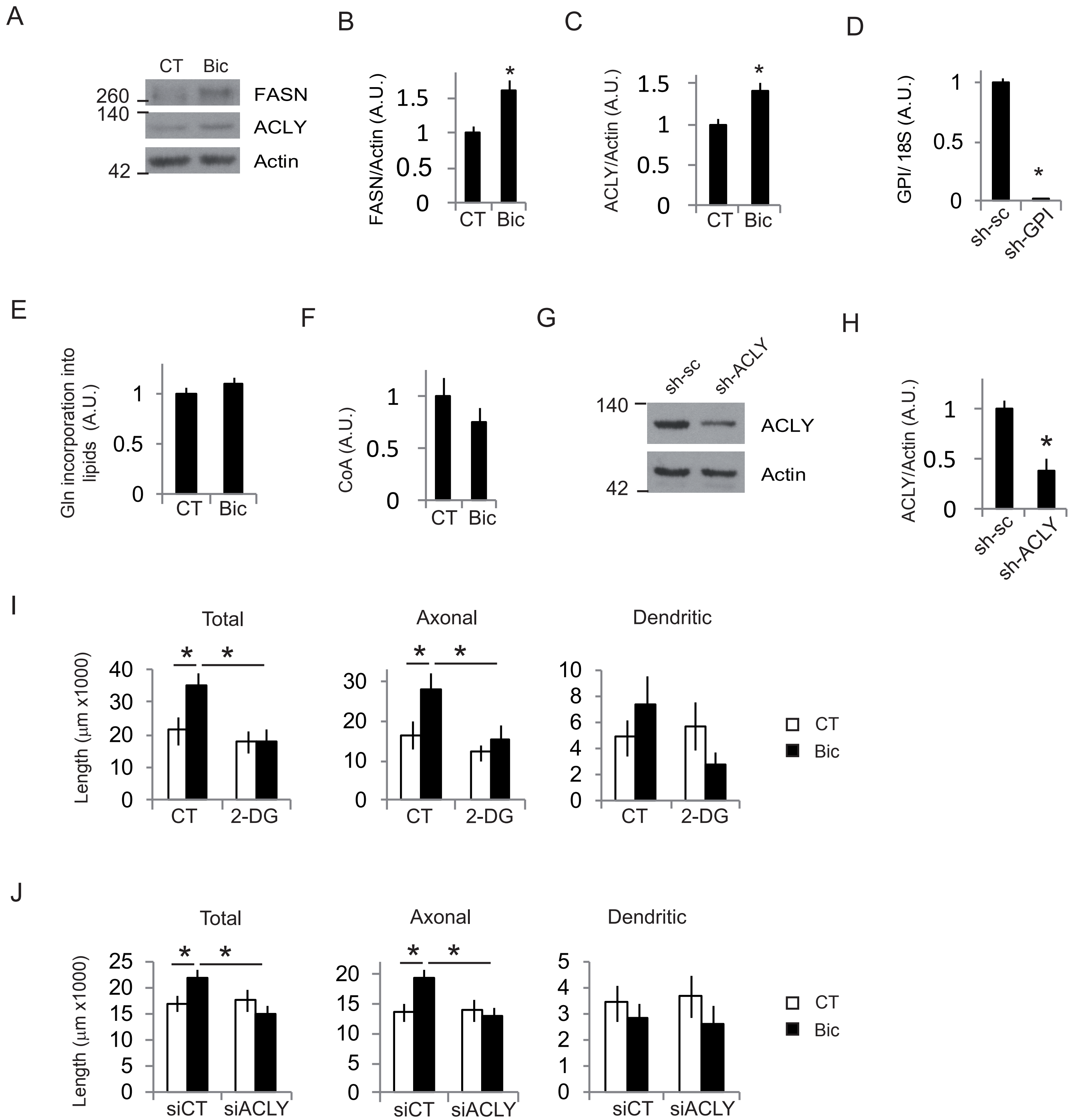
Soriano FX, Léveillé F, Papadia S, Higgins LG, Varley J, Baxter P, Hayes JD, Hardingham GE (2008) Induction of sulfiredoxin expression and reduction of peroxiredoxin hyperoxidation by the neuroprotective Nrf2 activator 3H-1,2-dithiole-3-thione. *Journal of Neurochemistry* **107:** 533-543

Soro-Arnaiz I, Li Qilong Oscar Y, Torres-Capelli M, Meléndez-Rodríguez F, Veiga S, Veys K, Sebastian D, Elorza A, Tello D, Hernansanz-Agustín P, Cogliati S, Moreno-Navarrete Jose M, Balsa E, Fuertes E, Romanos E, Martínez-Ruiz A, Enriquez Jose A, Fernandez-Real Jose M, Zorzano A, De Bock K, et al. (2016) Role of Mitochondrial Complex IV in Age-Dependent Obesity. *Cell Reports* **16:** 2991-3002

Zhang SJ, Steijaert MN, Lau D, Schutz G, Delucinge-Vivier C, Descombes P, Bading H (2007) Decoding NMDA receptor signaling: identification of genomic programs specifying neuronal survival and death. *Neuron* **53:** 549-562

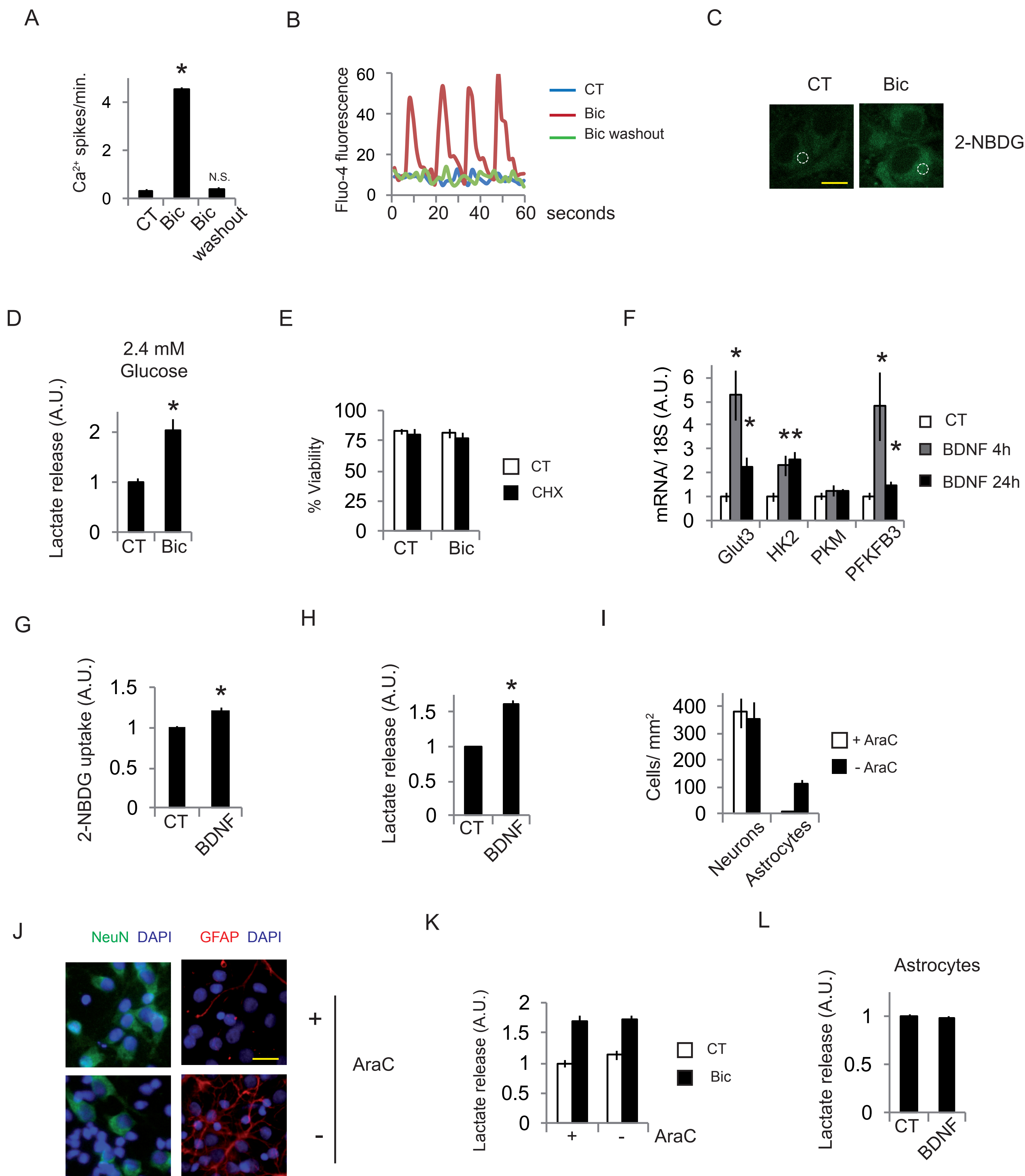
3. Appendix Figures

Appendix Figure S1 (related to figure 1)



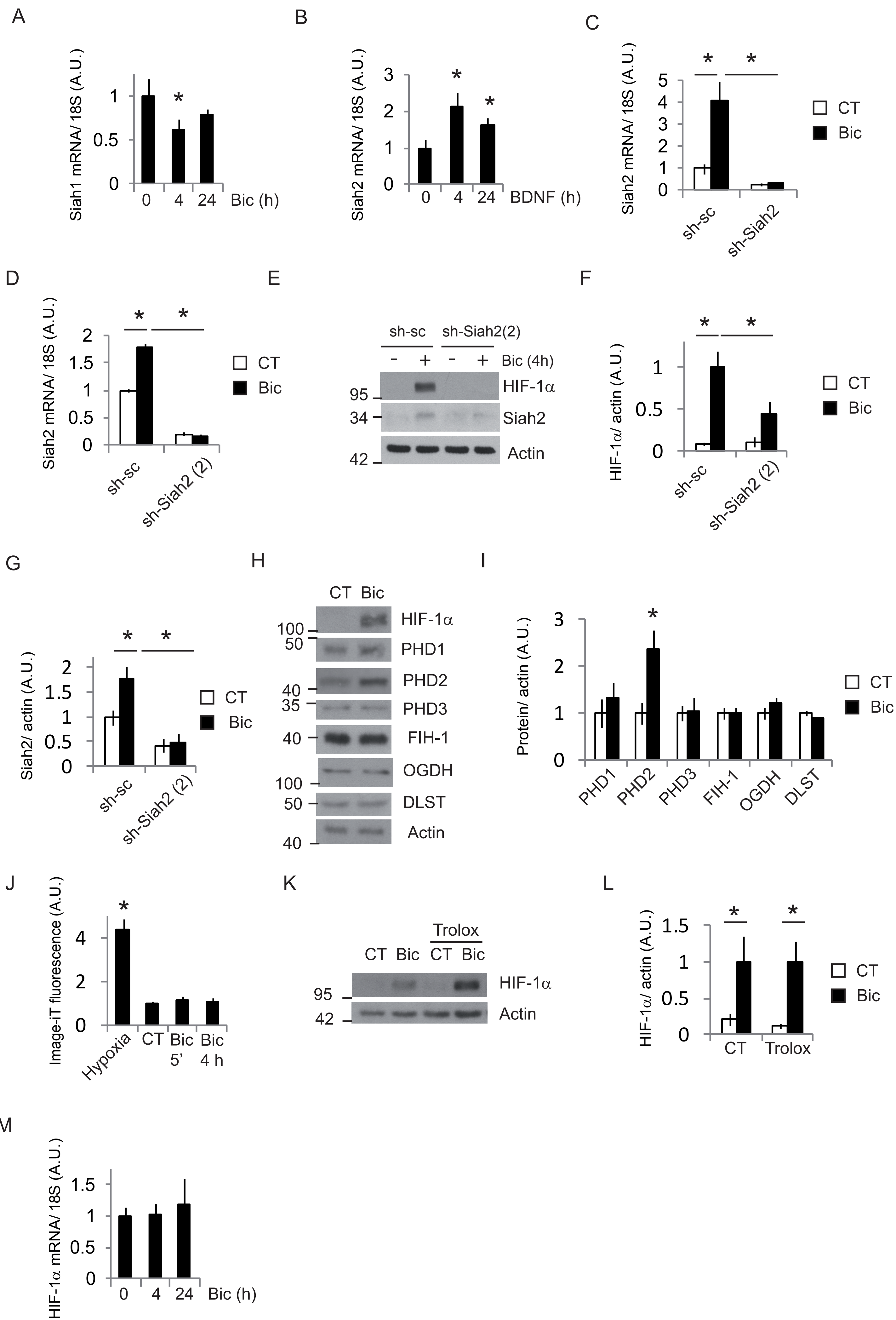
Appendix Figure S1. A) Cortical neurons were stimulated with Bic+4-AP for 4 hours and the indicated proteins were analyzed by western blotting. B and C) Densitometric analysis of the indicated proteins (n= 5 independent experiments). Values represent mean ± s.e.m. *p<0.05, two-tailed Student's t-test. D) GPI mRNA levels analyzed by qPCR of cortical neurons transduced with AAV expressing shRNA control (sh-sc) or targeting GPI (shGPI) (n= 4 independent experiments). Values represent mean ± s.e.m. *p<0.05, two-tailed Student's t-test. E) Neurons were incubated with ^{3}H-glutamine were stimulated with Bic+ 4-AP for 48 hours or left unstimulated (CT). Cellular lipids were extracted and radioactive counts measured (n= 4independent experiments). Values represent mean ± s.e.m. F) Determination of CoA levels after 24 hours Bic+4-AP stimulation (n= 4 independent experiments). Values represent mean ± s.e.m. G) Protein extracts of cortical neurons transduced with AAV expressing shRNA control (sh-sc) or targeting ACLY (shACLY) were analyzed by western blot and (H) densitometric analysis (n= 4 independent experiments). Values represent mean ± s.e.m. *p<0.05, two-tailed Student's t-test. I) Neurite length of control and Bic+4-AP stimulated neurons (for 48 hours) cultured in 25 mM of the non-metabolizable glucose analog 2-DG (n= 15 neurons from 3 independent experiments). Values represent mean ± s.e.m. *p<0.05, one-way ANOVA followed by Tukey's post-hoc test. J) Neurite length of control and Bic+4-AP stimulated neurons (for 48 hours) after transfection with non-targeting (siC) or ACLY-targeting (siACLY) siRNAs (n= 27-40 neurons from 6 independent experiments). Please, note that the siCT is the same that the shown in Fig. 6C since these experiments were done at the same time. Values represent mean ± s.e.m. *p<0.05, one-way ANOVA followed by Tukey's post-hoc test.

Appendix Figure S2 (related to figure 2)



Appendix Figure S2. A) Fluo-4 determination of Ca2+ spikes in neurons unstimulated or stimulated for 1 minute or for 24 hours and washed for 30 minutes, to allow restoration of ionic gradients. B) Representative tracings. C) Representative images showing 2-NBDG uptake. The white circle represents a selected ROI to be analyzed. Scale bar, 5 μm. D) Lactate released into the medium by neurons stimulated with Bic+4-AP (for 24 h) in medium containing 2.4 mM glucose (n= 4 independent experiments). Values represent mean ± s.e.m. *p<0.05, two-tailed Student's t-test. E) Neuronal viability of neurons treated with cycloheximide (10 μM) for 24 h. (n= 4 independent experiments). Values represent mean ± s.e.m. F) Cortical neurons were stimulated with BDNF (25 ng/ml) for 4 or 24 hours and mRNA expression of the indicated genes was determined by real-time qPCR (n= 3-6 independent experiments). Values represent mean ± s.e.m. *p<0.05, two-tailed Student's t-test. G) 2-NBDG uptake over 15 minutes in control or BDNF treated neurons (for 24 h) after washing and medium replacement (n= 4 independent experiments). Values represent mean ± s.e.m. *p<0.05, two-tailed Student's t-test. H) Neurons treated were stimulated with BDNF (25 ng/ml; for 24 h) before measuring the amount of lactate released into the medium (n= 4 independent experiments). Values represent mean ± s.e.m. *p<0.05, two-tailed Student's t-test. I) Quantification of glial and neuronal content in mixed cortical cultures treated or not with AraC (n= 3 independent experiments). Values represent mean ± s.e.m. J) Immunodetection of the indicated proteins in mixed cortical cultures treated or not with AraC. Scale bar, 25 μm. K) Lactate release after 24 hours of Bic+4-AP stimulation in mixed cultures with different proportions of astrocytes (n= 4 independent experiments). Values represent mean ± s.e.m. *p<0.05, one-way ANOVA followed by Tukey's post-hoc test. L) Lactate release in the medium of pure astrocyte culture treated for 24 hours with BDNF (25 ng/ml) (n= 3-7 independent experiments). Values represent mean ± s.e.m. *p<0.05, two-tailed Student's t-test

Appendix Figure S3 (related to figure 4)



Appendix Figure S3. A) Neurons were stimulated with Bic+4-AP for the indicated times and Siah1 mRNA was analyzed by qPCR (n= 5 independent experiments). Values represent mean ± s.e.m., *$p<0.05$, two-tailed Student's t-test. B) Neurons were stimulated with BDNF (25 ng/mL) for the indicated times and Siah2 mRNA was analyzed by qPCR (n= 5 independent experiments). Values represent mean ± s.e.m., *$p<0.05$, two-tailed Student's t-test. C and D) Cortical neurons were transduced with AAV expressing non-targeting shRNA (sh-sc) or targeting Siah2 at two different sequences (sh-Siah2), stimulated for 4 hours with Bic+4-AP and the mRNA expression was analyzed by qPCR (n= 6 independent experiments). Values represent mean ± s.e.m. *$p<0.05$, one-way ANOVA followed by Tukey post-hoc test. E) Representative western blot and (F and G) densitometric analysis of the indicated proteins. Cortical neurons were transduced with AAV expressing non-targeting shRNA (sh-sc) or targeting Siah2 (sh-Siah2(2)) and stimulated for 4 hours with Bic+4-AP. (n= 4 independent experiments). Values represent mean ± s.e.m. *$p<0.05$, one-way ANOVA followed by Tukey post-hoc test. H) Representative western blot of the indicated proteins and (I) densitometric analysis of protein samples from control and Bic+4-AP-stimulated (24 hours) (n= 3-4 independent experiments). J) Analysis of Image-iT fluorescence of neurons subjected to hypoxia for 1 hour or in normoxia unstimulated (CT) or stimulated with Bic+4-AP for the indicated times(n= 3-7 independent experiments). Values represent mean ± s.e.m. *$p<0.05$, two-tailed Student's t-test. K) Representative western blot and (L) densitometric analysis of neurons stimulated for 4 hours with Bic+4-AP in absence or presence of 100 μM trolox. (n= 4 independent experiments). Values represent mean ± s.e.m. Synaptic activity does not causes increased HIF-1α mRNA expression. M) Neurons were stimulated with Bic+4-AP for the indicated times and HIF-1α mRNA was analyzed by qPCR (n= 3 independent experiments). Values represent mean ± s.e.m.