Supporting Information

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SI Materials and Methods

Ex Utero Electroporation and Organotypic Slice Culture. Briefly, a solution containing 1 μg/μL of endotoxin-free plasmid DNA and 0.5% Fast Green as a loading dye (Sigma; 1:20 ratio) was microinjected into the lateral ventricles of isolated E15.5 embryonic mouse heads that were decapitated and placed in complete HBSS medium [1× HBSS solution (Gibco), 2.5 mM Hepes pH 7.4, 30 mM D-glucose, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 4 mM NaHCO3]. Plasmid DNA was then introduced into radio-glial telencephalic progenitors of the ventricular zone by electroporation with gold-coated electrodes using an ECM 830 electroporator (BTX) and the following parameters: five 100-ms long pulses separated by 100-ms long intervals at 30 V. For slice culture, the brain was extracted immediately after electroporation, embedded in 3% low-melting agarose, and vibratome sectioned (250-μm thick sections) using a Leica VT1000S vibratome. The resulting slices were cultured organotypically for 5 d using an air interface protocol on transwell inserts (1-μg pore size) coated with poly-D lysine (83 mg/mL; Sigma) and laminin (8.3 mg/mL), then fixed with 4% paraformaldehyde and stained for immunofluorescence. Dissociated E15.5 cortical cultures were performed using a papain-based enzymatic dissociation method as previously described. A total of 1.25×10^5 dissociated cortical progenitors were plated on 12-mm glass coverslips coated with laminin and poly-L lysine and cultured in serum-free culture medium (NeuroBasal medium supplemented with L-glutamine and penicillin/streptomycin, plus B27 and N2 supplements). Neuronal cultures were ultimately fixed with 4% paraformaldehyde and stained for immunofluorescence.

Western Blotting. Cells were scraped via cell lifter (Corning), harvested in culture medium, and centrifuged at 1,000 RPM for 5 min. Pellet was washed two times in cold DPBS and lysed in ice-cold lysis buffer containing 25 mM Tris (pH7.5), 2 mM $MgCl_2$, 600 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, and 1× protease and phosphatase mixture inhibitors (Sigma). Aliquots of the proteins were separated on 4–12% NuPAGE BisTris and then transferred to a polyvinylidene difluoride (PVDF) membrane. After transfer, the membrane was washed three times in TBS, blocked for 1 h at room temperature in 5% BSA in TBS, followed by 4 °C overnight incubation with appropriate primary antibody in 5% BSA-TBS. Western blot analysis was performed at 1:1,000 dilution of all primary antibodies with the following exception: anti-α tubulin (1:15,000). Next day, the membrane was washed three times in TBS-Tween, incubated at room temperature for 1 h with IRDye infrared secondary antibody (LI-COR Biosciences) at 1:2,000 dilution in 5% BSA-TBS, followed by 2× TBS-T and 1× TBS washes. Scanning, analyzing, and quantification of blots were performed via the Odyssey Infrared Imaging System (Fig. S1 *D–G*) or Alpha Innotech FluorChemQ (all others). Three or more independent experiments were performed for all immunoblotting data. Quantification data are represented by bar graphs with error bars that indicate the SEM.

Constructs and Reagents. The following vectors were used (percentages indicate the knockdown level indicated by Sigma-Aldrich for validated shRNA vectors, when the information is available): TRCN0000113949 (70%) and TRCN0000113950 for Tsc1 and TRCN0000042723 (71%) and TRCN0000042724 (91%) for Tsc2. For each gene, shRNA coding plasmids were pooled to a final concentration of 1 μg/μL (1:1 ratio) and co-transfected with GFP-coding pCIG2 vector (0.5 μg/μL final concentration).

Immunofluorescent Staining. Dissociated neuronal cultures and organotypic slice cultures were fixed with 4% paraformaldehyde for 30 min. Postnatal mouse brains were fixed by intracardiac perfusion of 4% paraformaldehyde after anesthesia with avertin. Following perfusion, brains were quickly dissected and postfixed in 4% paraformaldehyde for 60 min, then sectioned (50-μm thick sections) as described above. Following fixation, samples were washed with PBS three times and then permeabilized in blocking buffer (1× PBS, 5% BSA, 0.05% Triton X-100) for 1 h. Incubation with primary antibodies was performed overnight with the indicated antibodies diluted in blocking buffer supplemented with 10% goat serum. After three washes with 1× PBS, the samples were stained with the appropriate Alexa conjugated secondary antibodies (Molecular Probes; 1:2,000) for 1 h. Cells were then washed in PBS and slides were mounted. Nuclear DNA was stained with DRAQ5 dye (Alexis; 1:10,000). Fluorescent immunostaining was observed using a LEICA TCS-SL laser scanning confocal microscope equipped with an Argon laser (488 nm), green Helium-Neon laser (546 nm), and red Helium-Neon laser (633 nm) for observation of Alexa-488/GFP, Alexa-546, and Alexa-647/DRAQ5, respectively.

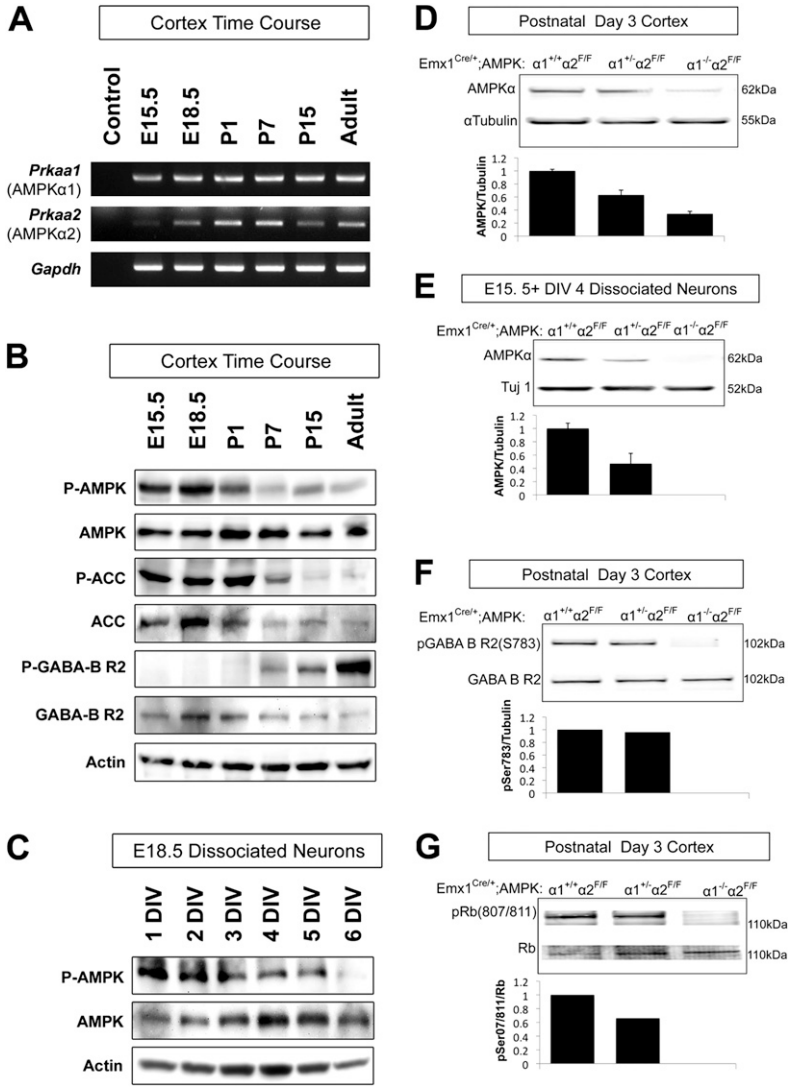

Fig. S1. Expression of AMPKα1 and AMPKα2 in the developing cortex. (*A*) Expression profile of AMPKα1 and AMPKα2 at various developmental stages. RT-PCR on total mRNA extracted from mouse cortex. The housekeeping mRNA Gapdh was used as an internal control. The control lane represents a RT-PCR performed with no template RNA. (*B*) Expression and activity of AMPKα proteins in the mouse cortex. The catalytic activity of the AMPK enzyme was determined by the phosphorylation of AMPKα on threonine 172 residue (p-AMPK), and by the phosphorylation of its downstream targets ACC and $GABA_B$ R2. Actin expression was used for normalization. (*C*) Expression and activity of AMPKα proteins in cortical neurons cultured in vitro. Cortical neurons were grown in vitro after dissociation in conditions allowing their progressive polarization, and collected every day. Western blot analysis was performed to monitor the expression level of total and phosphorylated AMPKα. Actin was used as a normalization control. (*D*–*G*) In vivo genetic ablation of AMPKα1 and AMPKα2 in the developing cortex. Western blot analysis reveals decreased levels of total AMPKα (1 and 2) in cortical lysates from $Emx1^{Cre/+}$; $AMPK\alpha1^{-/-}$; $AMPK\alpha2^{F/F}$ mice compared with control littermates in cortical tissue (*D*) and dissociated cortical neurons (*E*). Ablation of AMPKα in the cortex results in decreased phosphorylation of $GABA_B$ R2 at residue Ser783 (*F*) as well as the retinoblastoma tumor suppressor gene Rb phosphorylated at Ser807/811 (*G*). Bar graphs represent quantification of indicated protein relative to the nonphosphorylated form of the protein (when appropriate) and/or to α-tubulin or neuron-specific β-tubulin-III (TuJ1). Error bars represent SE to the mean (n = 3).

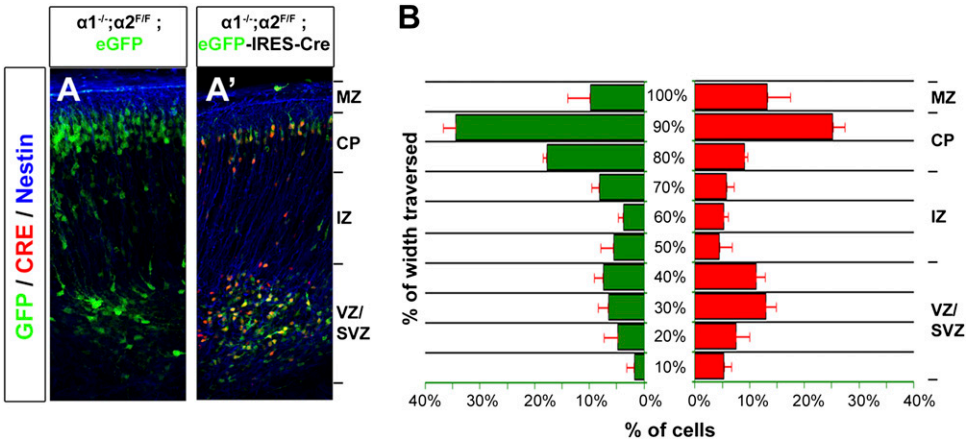

Fig. S2. AMPKα is not required for radial migration of cortical neurons. (*A* and *A′*) E15.5 cortical slices of AMPKα1$^{-/-}$; AMPKα2$^{F/F}$ brains were cultured for 5 d after electroporation with EGFP (control, *A*) or Cre-IRES-EGFP (experimental, *A′*). Slices were stained with anti-nestin antibody revealing radial glial scaffold (blue). MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone; SVZ, subventricular zone. (*B*) Quantification of three independent experiments of cell distribution for slices expressing EGFP (green bars, *Left*) or Cre-IRES-EGFP (red bars, *Right*).

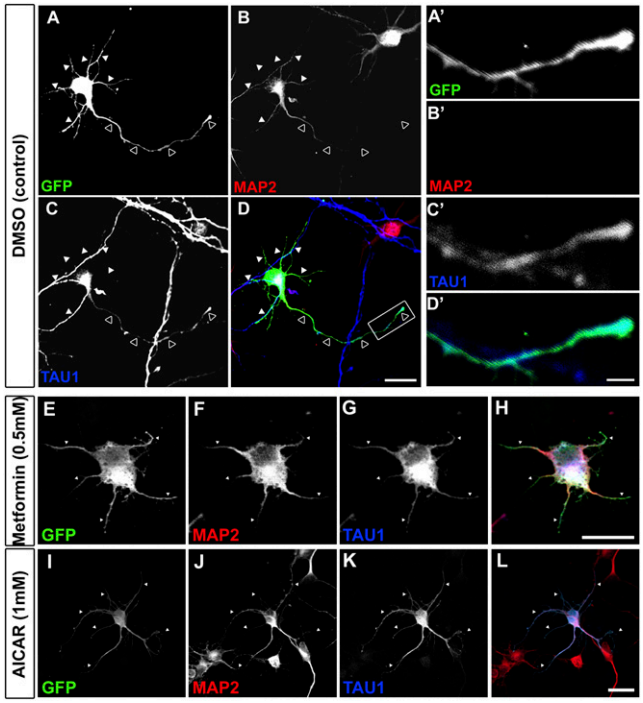

Fig. S3. AMPK activators alter cortical neuron morphology. (*A–D*) Examples of E15.5 cortical neurons electroporated, dissociated, and grown in vitro for 4 d. Neurons were treated for 3 d with vehicle (DMSO) starting at 1 div. Neuron morphology was visualized by GFP expression (*A*). Neurons were stained with the somato-dendritic marker MAP2 (*B*) and the axon marker Tau1 (*C*). White arrow: MAP2 positive, Tau1 negative dendrites. Open arrow: MAP2 negative, Tau1 positive axon. (Scale bar, 25 μm.) (*A′–D′*) Higher magnification of the boxes from *D*. (Scale bar, 3 μm.) (*E–L*) Morphology of neurons treated for 3 d with metformin (0.5 mM) (*E–H*) or AICAR (1 mM) (*I–L*). Neurons were immunostained with GFP (*A*), MAP2 (*B*), and Tau1 (*C*). White arrow: MAP2 and Tau1 positive neurites. (Scale bar, 20 μm.)

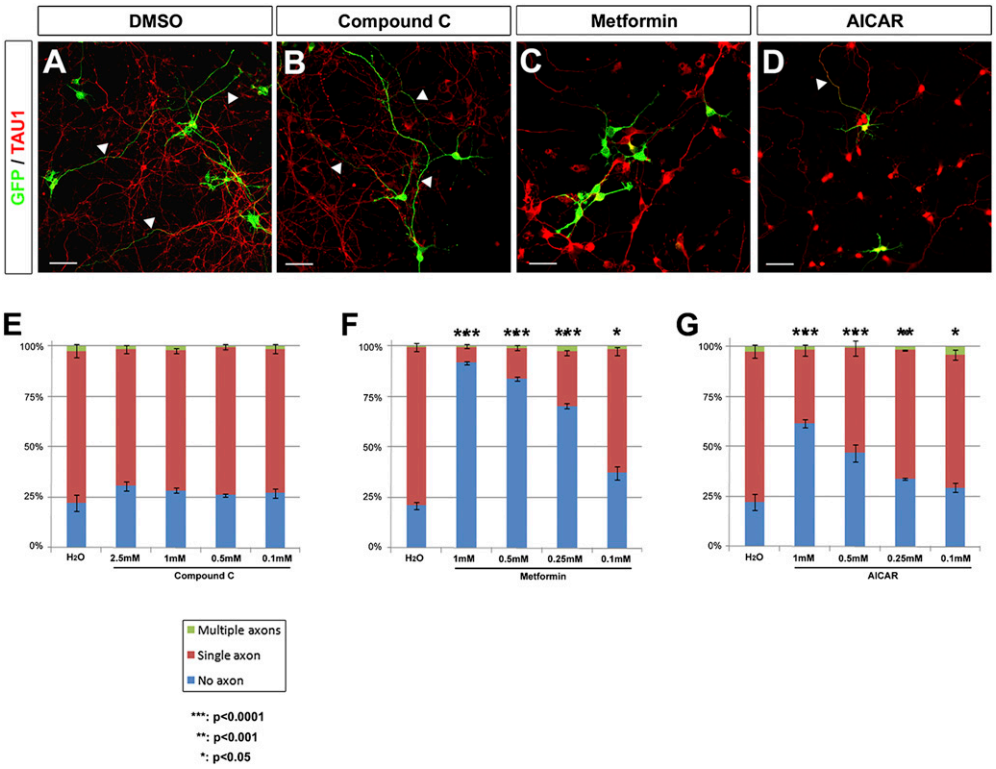

Fig. S4. Metabolic stress impairs axogenesis. (*A–D*) E15.5 cortical neurons electroporated, dissociated, and maintained for 4 div. At 1 div, cells were treated for 3 d with either vehicle (H_2O or DMSO) (*A*), the AMPK inhibitor compound C (5 μM) (*B*), or the AMPK activators metformin (0.5 mM) (*C*) and AICAR (1 mM) (*D*). Neurons grown in control conditions or treated with compound C exhibit a single axon (arrowhead). Note the absence of axons in neurons cultured in the presence of AICAR and metformin, which activates AMPK. (Scale bar, 50 μm.) (*E–G*) Dose-dependent effect of the AMPK inhibitor, compound C (*E*), or activators metformin (*F*) and AICAR (*G*) on neuronal polarity. E15.5 neurons were treated at 1 div for 3 d with the indicated drugs at the indicated concentrations. Quantifications are based on ≥100 neurons per condition. Mean proportion represents neurons with no (blue), one (red), or multiple (green) >100 μm long and Tau1-positive axon for at least three independent experiments. Fisher's exact test was used to statistically analyze the data represented (***$P < 0.001$; *$P < 0.05$).

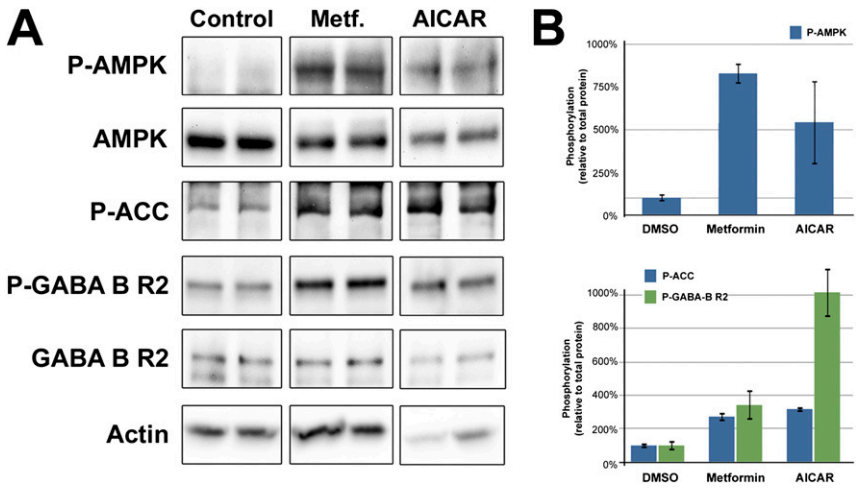

Fig. S5. AMPK activators metformin and AICAR increase AMPK activity in cortical neurons. Western blot analysis after E18.5 dissociated cortical neurons cultured for 1 d in vitro after treatment with metformin (2 mM) or AICAR (2 mM) for 3 consecutive days. Both treatments increased the phosphorylation of AMPKα (Thr172, compare with total AMPKα expression). Treatments also increased the phosphorylation of two downstream targets of AMPK, ACC (Ser79) and $GABA_B$ R2 (Ser783). Actin was used as normalization control.

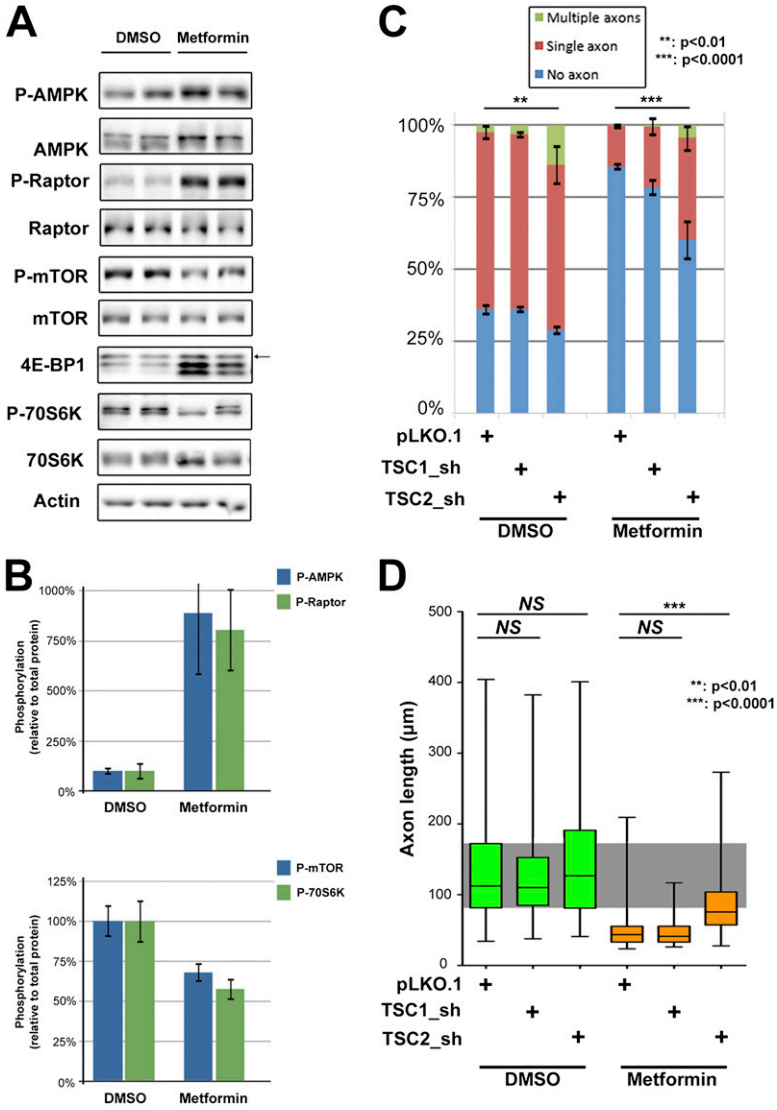

Fig. S6. TSC1 and TSC2 knockdown only partially rescues metformin phenotype. (*A*) Western blot analysis after in vitro culture of E18.5 mouse cortical neurons cultured for 1 d, then treated with metformin (2 mM) for 3 d in vitro. Phosphorylation of AMPK (phospho-Thr172), raptor (phospho-Ser792), mTOR (phospho-Ser2448), p70S6K (phospho-Ser371), and 4E-BP1 were assessed similarly to what is described in Fig. 4. (*B*) Quantification of phospho-AMPK, phospho-raptor, phospho-mTOR, and phospho-70S6K signal were normalized to total amount of corresponding proteins compared with control condition (DMSO vehicle only). Error bars represent SEM ($n = 5$). (*C* and *D*) Quantification of neuronal polarity (*C*) and longest neurite length (*D*) for five independent experiments ($n \geq 500$ for polarization, $n \geq 500$ for length). Statistical analyses were performed using Fisher's exact test (*C*) or one-way ANOVA (*D*).

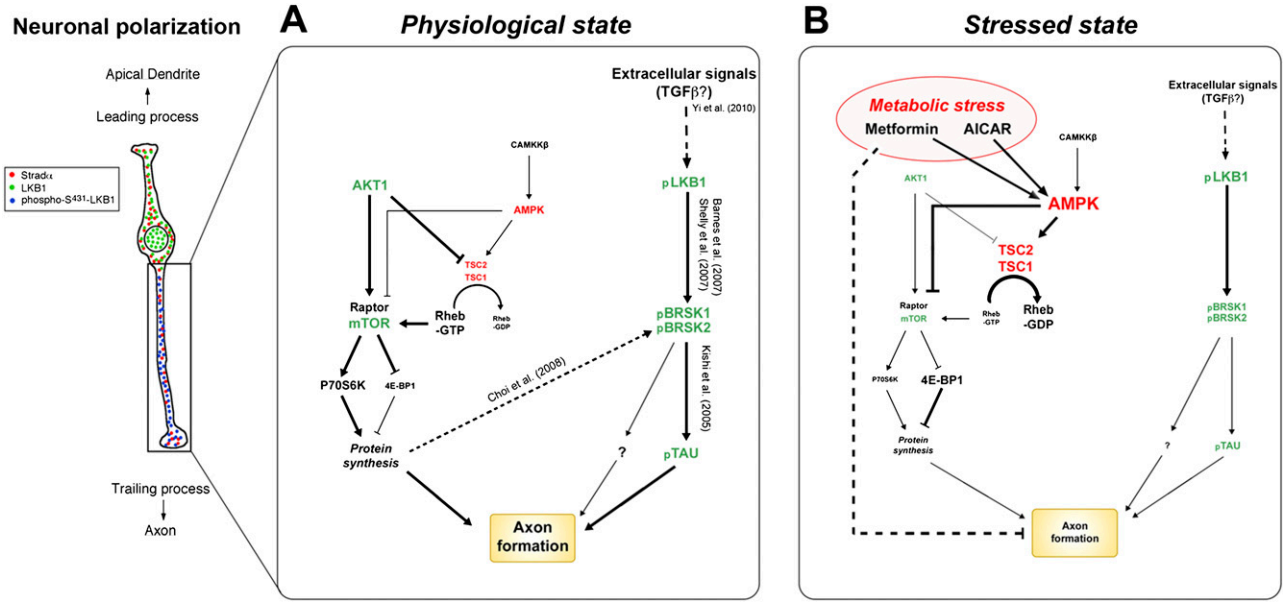

Fig. S7. Model: AMPK overactivation impairs axogenesis during metabolic stress. Synthesis of pathways demonstrated or previously shown to regulate axogenesis both under normal physiological conditions (*A*) or (metabolic) stress-induced states (*B*). Both positive (green) and negative (red) regulators of axon outgrowth intersect to determine net axon outgrowth/specification. During normal mammalian development in vivo (*A*), axon specification and subsequent axon growth requires local phosphorylation of LKB1 on S431 (1, 2) to specify one neurite (future trailing process) to become the axon. This local phosphorylation might be triggered by proteins such as cAMP-dependent kinase (PKA) or other kinases in response to graded extracellular cues such as TGF-β (3). LKB1 phosphorylates and activates AMPK-like proteins such as BRSK1/2 (SAD-A/B) and probably others, which in turn phosphorylate key effectors such as the microtubule-associate protein Tau and probably many other effectors that participate in axon formation (1). Another parallel pathway involving the kinase AKT and AMPK acts as positive and negative regulators of mTOR and local protein synthesis, respectively. AKT has previously been shown to act as a promoter of axogenesis, whereas TSC1/2 have been shown to act as negative regulators of axogenesis in part through mTOR-mediated translation of SAD-A/B (BRSK2/1; ref. 4). Our results show that AMPK is not required constitutively for axon formation or neuronal differentiation but AMPK overactivation under conditions of metabolic stress (mimicked by AICAR or metformin treatments) results in overactivation of TSC2 and inhibition of raptor, which leads to inhibition of axogenesis. See text for further details.

1. Barnes AP, et al. (2007) LKB1 and SAD kinases define a pathway required for the polarization of cortical neurons. *Cell* 129:549–563.
2. Shelly M, Cancedda L, Heilshorn S, Sumbre G, Poo MM (2007) LKB1/STRAD promotes axon initiation during neuronal polarization. *Cell* 129:565–577.
3. Yi JJ, Barnes AP, Hand R, Polleux F, Ehlers MD (2010) TGF-beta signaling specifies axons during brain development. *Cell* 142:144–157.
4. Choi YJ, et al. (2008) Tuberous sclerosis complex proteins control axon formation. *Genes Dev* 22:2485–2495.

Table S1. List of antibodies and concentration used

Target protein	Species	Company	Concentration used
AMPKα	Rabbit	Cell Signaling	1:1,000 (WB)
P-AMPKα (T172)	Rabbit	Cell Signaling	1:1,000 (WB)
Tuj1 (βIII-tubulin)	Mouse	Sigma	1:1,000 (WB, ICC)
$GABA_B$ R2	Rabbit	Cell Signaling	1:1,000 (WB)
$GABA_B$ R2 (S783)	Rabbit	PhosphoSolutions	1:1,000 (WB)
Rb	Mouse	Santa Cruz	1:1,000 (WB)
Rb (S807/811)	Rabbit	Santa Cruz	1:1,000 (WB)
α-Tubulin	Mouse	Sigma	1:15,000 (WB)
CTIP 2	Mouse	Abcam	1:500 (IHC)
Cux1	Rabbit	Santa Cruz	1:500 (IHC)
Tbr1	Rabbit	Chemicon	1:500 (IHC)
Nestin	Mouse	BD-Bioscience	1:2,000 (IHC)
Activated caspase-3	Rabbit	Cell Signaling	1:500 (IHC)
Tau-1	Mouse	Chemicon	1:2,000 (ICC)
MAP2 (a/b isoforms)	Rabbit	Millipore	1:1,000 (IHC); 1:2,000 (ICC)
GFP	Chicken	Upstate	1:1,000 (ICC, IHC)
Cre	Rabbit	Novagen	1:1,000 (IHC)
P-raptor (S792)	Rabbit	Cell Signaling	1:1,000 (WB)
Raptor	Rabbit	Cell Signaling	1:1,000 (WB)
P-mTOR (S2448)	Rabbit	Cell Signaling	1:1,000 (WB)
mTOR	Rabbit	Cell Signaling	1:1,000 (WB)
4E-BP1	Rabbit	Cell Signaling	1:2,000 (WB)
P-70S6K (S371)	Rabbit	Cell Signaling	1:1,000 (WB)
70S6K	Rabbit	Cell Signaling	1:1,000 (WB)
Actin	Mouse	Millipore	1:10,000 (WB)
P-ACC	Rabbit	Cell Signaling	1:1,000 (WB)
ACC	Rabbit	Cell Signaling	1:1,000 (WB)

WB, Western blot; ICC, immunocytochemistry; IHC, immunohistochemistry.