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Supplemental Information

Metformin Acts on Two Different Molecular Pathways to Enhance Adult Neural Precursor Proliferation/Self-Renewal and Differentiation

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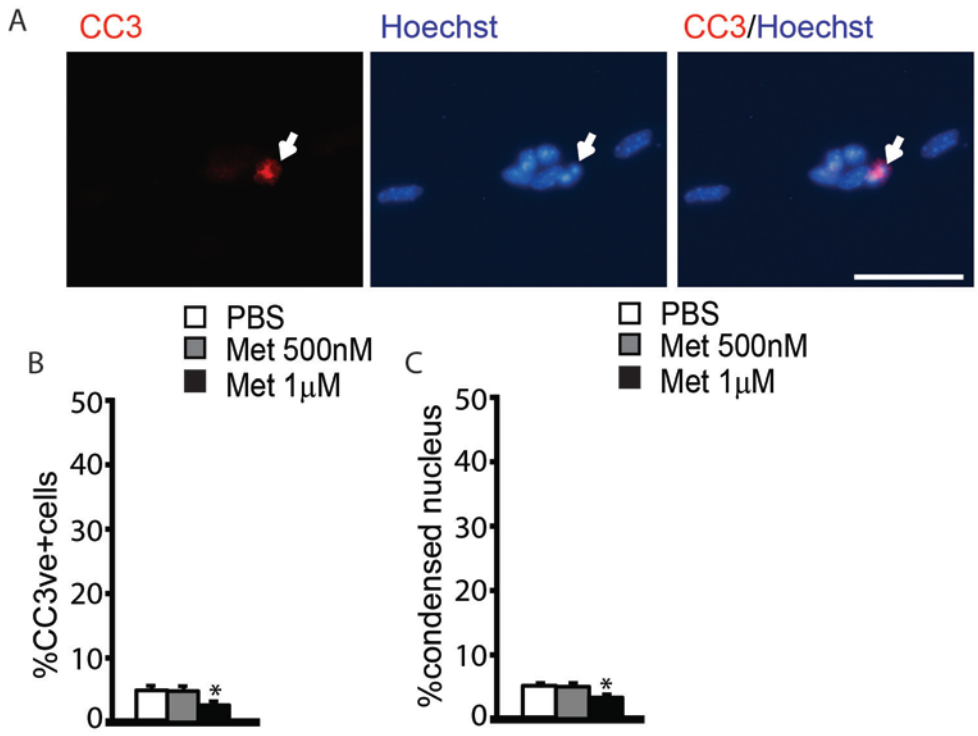

Figure S1. Metformin enhances adult NPC survival in a dose-dependent manner, related to Figure 1. (A-C) monolayer adult NPCs were cultured in proliferating SFM in the presence or absence of 500nM-1uM metformin and quantified 2 days later, as pictured in (A). Scale bar = 100μm. (B) Quantification of the percentage of cleaved caspase 3 (CC3) following metformin or PBS exposure as in (A). * $p \leq 0.05$ (n = 3 for each group) (C) Quantification of the percentage of condensed nuclei following metformin or PBS exposure, as in (A). * $p \leq 0.05$ (n =3 for each group).

Supplemental Experimental Procedures:

BrdU labeling

Brains were post-fixed overnight in 4% PFA, dehydrated in 30% sucrose, and sectioned at 20 μm using a Leica CM1850 cryostat (Leica Biosystems, Concord, ON, Canada). BrdU immunostaining was performed as previously described (Wang et al., 2012). Sections were post-fixed with 4% PFA, incubated in 1 N HCl for 30 min at 60°C, blocked and permeabilized with 5% BSA and 0.3% Triton-X. Sections were incubated with anti-BrdU antibody at 4°C overnight, detected using an anti-rat secondary antibody, counterstained with Hoechst 33258 (1:2000, Sigma-Aldrich) and mounted with GelTol (Fisher).

Transfections

For overexpression of *CA-AMPKα1*, individual NPCs derived from secondary neurospheres were plated onto poly-L-Ornithine (Sigma) and laminin (BD)-coated wells in the presence of serum-free medium (SFM) containing 20 ng/ml EGF (Sigma-Aldrich), 10 ng/μl FGF2 (Sigma-Aldrich), 1x B27 (BD) and 2 μg/ml heparin (Sigma-Aldrich) at the density of 100,000 cells/well for 24-well plates. Then, 0.6 μg DNA (1:2 ratio of *PB-CAG-eGFP* versus *CA-AMPKα1 or Empty Vector* plasmids) and 1.8 μl TransIT-X2 (Mirus) were mixed with 50 μl DMEM/F12 medium, incubated for 30 min and added to NPCs 24 h after plating. 24h after transfection, the culture medium were changed to SFM containing 10% FBS for another 7 days. The rat *CA-AMPKα1* construct was purchased from addgenes (plasmid #27632) and subcloned into pcDNA3.1(+) backbone.

Quantitative RT-PCR

RNA was extracted from metformin- or PBS-exposed primary neurospheres using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, Norcross, GA) and was treated with DNase I using the

E.Z.N.A RNase-free DNase I Set (Omega Bio-Tek). cDNA was synthesized from 1µg total RNA using RevertAid H Minus M-MulV Reverse Transcriptase (Fermentas, Thermo Scientific, Waltham, MA, USA), and quantitative PCR was performed using Lightcycler 480 SYBR Green I Master mix (Roche, Laval, QC, Canada), following the manufacturer's instructions. The following primers were used for quantitative PCR: TAp73F – GCACCTACTTTGACCTCCCC, TAp73R – GCACTGCTGAGCAAATTGAAC, GAPDHF – GGGTGTGAACCACGAGAAATA, GAPDHR – CTGTGGTCATGAGCCCTTC. GAPDH mRNA was used as an endogenous control for all reactions, and all reactions were run in triplicate. Quantitative PCRs were run on C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA), and analyzed using Bio-Rad CFX Manager Software (Bio-Rad).

Immunocytochemistry, microscopy, and quantification

For immunocytochemistry, the cultured cells were fixed with 4% PFA, blocked, and permeabilized with 10% normal goat serum (NGS) and 0.3% Triton-X. Fixed cells were then incubated with primary antibodies at 4ºC overnight, with secondary antibodies at room temperature for 1 hour, counterstained with Hoechst 33258 (1:2000, Sigma-Aldrich) and mounted with GelTol (Fisher).

Digital image acquisition was performed using Zeiss Axioscope with AxioVision software (Zeiss). For quantification of precursor cells *in vivo*, hippocampi and SVZ regions were serially sectioned as previously described (Fujitani et al., 2010). BrdU-positive precursors in the hippocampi were counted in every 10th section throughout the entirety of the structure. Counts were then normalized by multiplying for the total number of sections. BrdU-positive precursors in the SVZ regions were counted in a similar manner as described for the hippocampus. Counts were then normalized by multiplying for the total number of sections. For quantification of

neuronal differentiation in culture, more than 400 cells per condition in at least 6 randomly chosen fields per experiment were counted. Statistical analyses were performed using a two-tailed Student's t test, unless indicated otherwise. In all cases, error bars indicate the standard error of the mean (SEM).

Antibodies

The primary antibodies used were rat anti-BrdU (1:200; Accurate Chemical, Cat# OBT0030G), mouse anti-βIII-tubulin (1:500; Covance, Cat# MMS-435P), rabbit anti-cleaved caspase 3 (1:1000; Cell Signaling Tech, Cat #9661), chicken anti-GFP (1:1000; Abcam, Cat#ab13970). The secondary antibodies used were Alexa Fluor 488-conjugated goat anti-rat IgG (1:1000; Cell Signaling Tech, Cat #4416), Alexa Fluor 555-conjugated goat anti-mouse IgG (1:1000; Cell Signaling Tech, Cat #4409), Alexa Fluor 488-conjugated goat anti-chicken IgG (1:1000; Life Tech, Cat #11039), Alexa Fluor 555-conjugated goat anti-rabbit IgG (1:1000; Cell Signaling Tech, Cat #4413).

References

Fujitani,M., Cancino, G.I., Dugani, C.B., Weaver, I.C., Gauthier-Fisher, A., Paquin, A., Mak, T.W., Wojtowicz, M.J., Miller, F.D. and Kaplan, D.R. (2010) TAp73 acts via the bHLH Hey2 to promote long-term maintenance of neural precursors. Curr. Biol. *20*, 2058–2065.