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Supplementary Information for

Dual-process brain mitochondria isolation preserves function and clarifies protein composition

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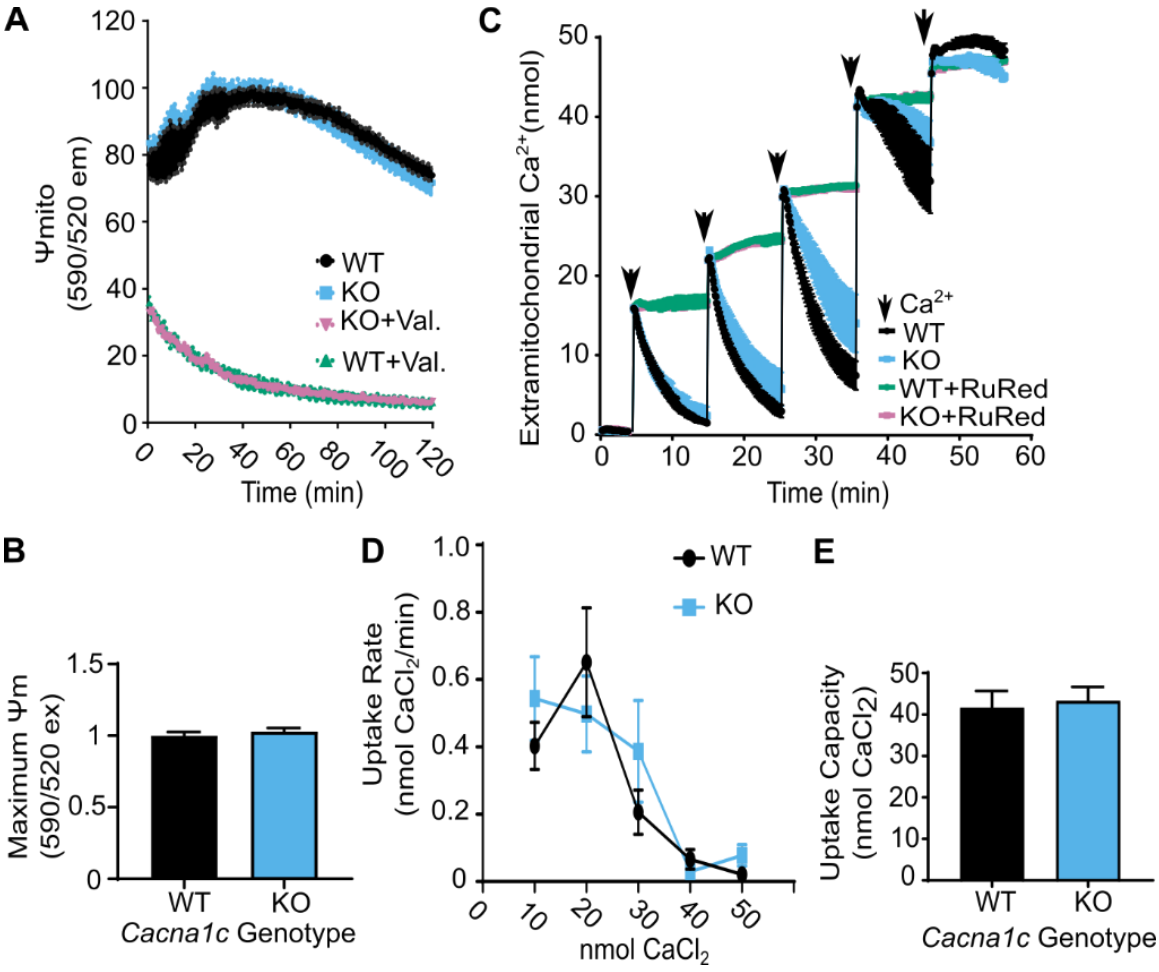

Fig. S1. Mitochondria from brain-*Cacna1c* KO mice are functionally normal. (*A*) JC-1 isolated mitochondrial membrane potential traces comparing maximum and maintained membrane potential in semi-pure mitochondria from brain-*Cacna1c* WT and KO mice show no significant differences. Valinomycin (Val) served as a negative control. SEM is represented by line weight (n=4, males). (*B*) Maximum attained mitochondrial membrane potential for mitochondria purified from WT and KO mice (n=4, males). (*C*) Representative Ca^{2+} uptake capacity traces from semi-pure mitochondria from brain-*Cacna1c* WT and KO mice show no significant differences. Increased fluorescence at 531 nm indicates increased extramitochondrial Ca^{2+}. Ruthenium red (RuRed) served as a negative control. 10 nmol Ca^{2+} additions are represented by black arrows. Line weight represents SEM (n=4-8, males and females, combined from two experiments). (*D*) Ca^{2+} uptake rate, calculated by nonlinear regression, between brain-*Cacna1c* WT and KO mice after sequential Ca^{2+} addition shows no difference. (*E*) Ca^{2+} uptake capacity between brain-*Cacna1c* WT and KO mice, identified as Ca^{2+} addition that failed to elicit a negative slope for extramitochondrial fluorescence, show no significant difference.

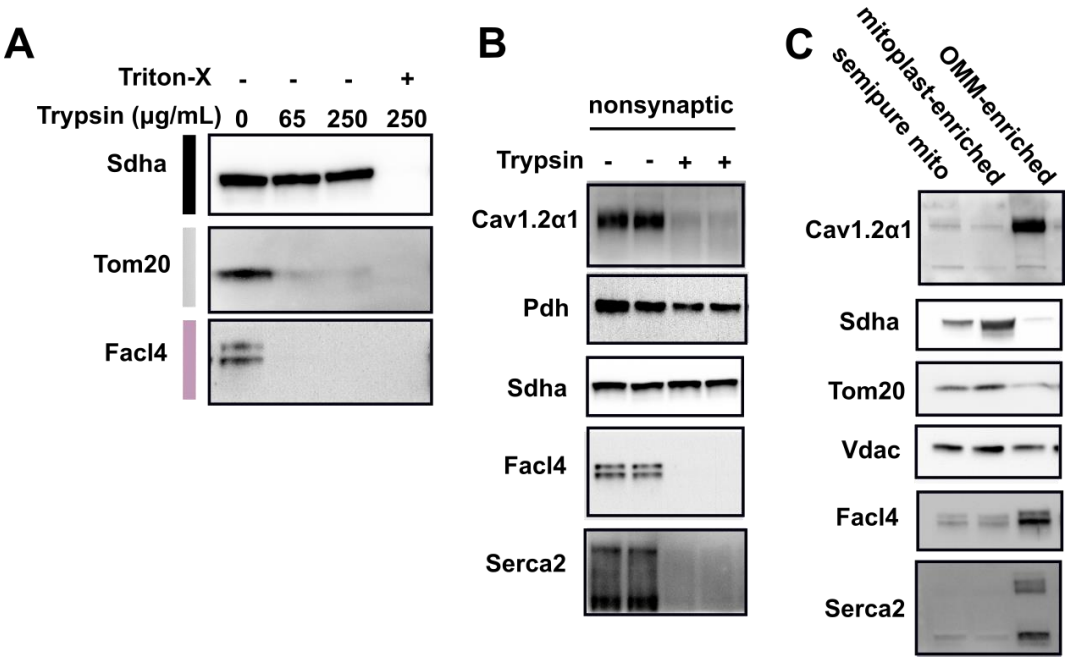

Fig. S2. Trypsin digest optimization for outer mitochondrial membrane (OMM) digest, and French Press fractionation. (A) Nonsynaptic mitochondria were digested with varying concentrations of trypsin and tested for digest of OMM and MAM proteins (Tom20 and FACL4), with preservation of inner mitochondrial membrane protein (IMM) Sdha. Triton X was used as a positive control for protein accessibility (repeated with n=6, combined from three experiments). (B) Supplemental digests of nonsynaptic brain mitochondria shows digestion of MAM proteins (Facl4 and Serca2), with preservation of IMM protein Sdha and matrix protein Pdh E1α, along with depletion of Cav1.2α1. (C) Supplemental French press fractionation of nonsynaptic brain mitochondria shows enrichment of $Ca_v1.2\alpha1$ in OMM-enriched fractions, which were also enriched for Vdac, FaclL4, and Serca2, while being replete of the IMM protein Sdha.

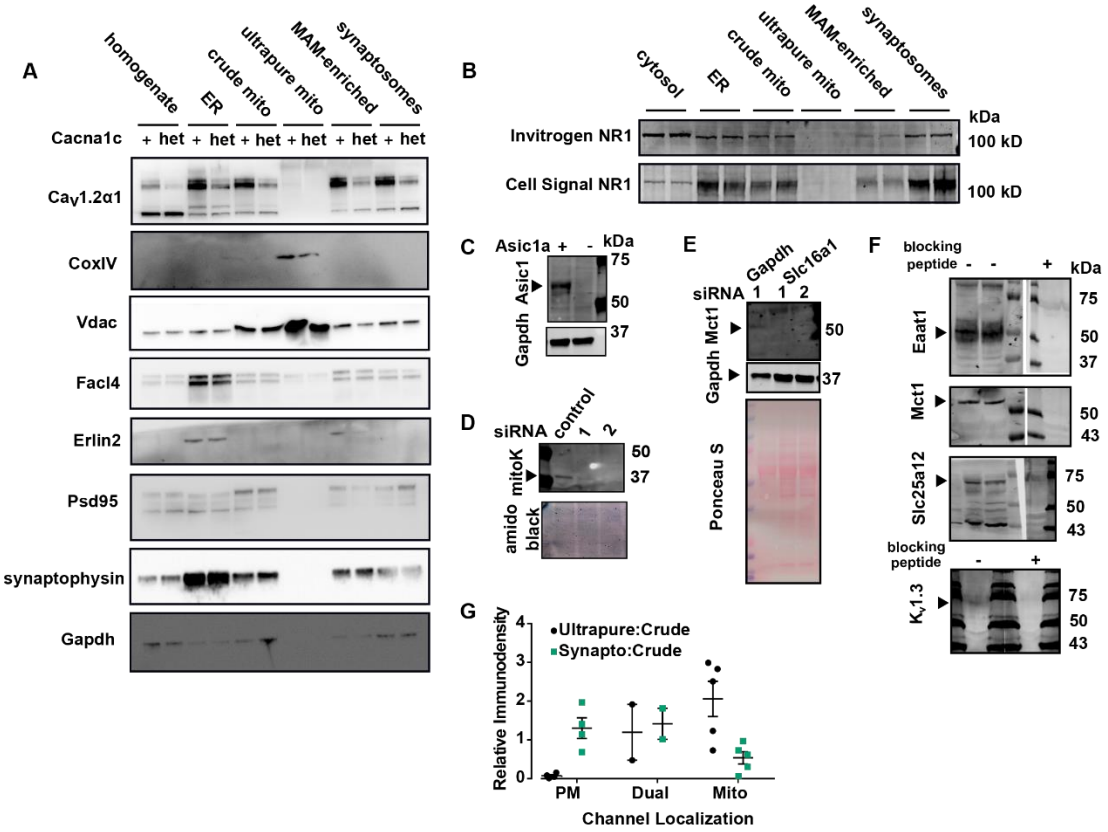

Fig. S3. Mitochondrial ultra-purification confirmation and divergent enrichment patterns of classical mitochondrial and plasma membrane proteins. (*A*) Western blots show mitochondria ultrapurification marker enrichment in homogenate, endoplasmic reticulum (ER), crude mitochondria (mito), ultrapure mito, MAM-enriched preparation, and synaptosomes. (*B*) Two different antibodies against NR1 show consistent depletion in ultrapurified mitochondria. (*C*) Asic1 KO brain lysates show loss of immunoreactivity at expected molecular weight. Gapdh was used as a loading control. (*D*) Lysates from Neuro2a cells treated with two different pairs of mitoK siRNAs compared to control show loss of immunoreactivity at expected molecular. Amido black total protein stain was used as a comparative control. (*E*) Lysates from Neuro2a cells treated with two different pairs of siRNAs against Mct1 and Gapdh show loss of protein immunoreactivity at expected molecular weights. Ponceau S was used as total protein control. (*F*) Antibody specificity for Eaat1, Mct1, Slc25a12, and $K_V1.3$ was confirmed by peptide blocking control on synaptosomal proteins with (+) or without (-) inclusion of blocking peptide prior to primary antibody incubation. (*G*) Relative enrichment patterns for plasma and mitochondrial membrane and dually localized proteins in subcellular fractions over enrichment in crude mitochondria. All antibody validation steps were done with an n=2 (males and females), combined from two experiments.

Gene	DsiRNA 1	DsiRNA 2
Ccdc51	mm.Ri.Ccdc51.13.1 rGrGrUrGrArUrArUrArArUrGrArArUrCrUrUrGrArGrGrUAC rGrUrArCrCrUrCrArArGrArUrUrCrArUrUrArUrArUrCrArCrCrArA	mm.Ri.Ccdc51.13.2 rGrUrGrArArCrArGrGrCrUrUrCrUrArGrCrUrArUrUrCrCCT rArGrGrGrArArUrArGrCrUrArGrArArGrCrCrUrGrUrUrCrArCrGrG
Slc16a1	mm.Ri.Slc16a1.13.1-SEQ1 rGrArA rUrGrA rGrUrU rUrCrA rArArU rCrArG rUrArC rArCT T rArArG rUrGrU rArCrU rGrArU rUrUrG rArArA rCrUrC rArUrU rCrArG	mm.Ri.Slc16a1.13.2 rGrGrA rArUrU rCrArU rCrUrA rCrArC rUrUrA rArArA rUrGC C rGrGrC rArUrU rUrUrA rArGrU rGrUrA rGrArU rGrArA rUrUrC rCrArA
Gapdh	mm.Ri.Gapdh.13.1 rGrGrU rCrCrC rArGrC rUrUrA rGrGrU rUrCrA rUrCrA rGrGT A rUrArC rCrUrG rArUrG rArArC rCrUrA rArGrC rUrGrG rGrArC rCrCrC	mm.Ri.Gapdh.13.2 rCrCrA rGrCrU rUrArG rGrUrU rCrArU rCrArG rGrUrA rArAC T rArGrU rUrUrA rCrCrU rGrArU rGrArA rCrCrU rArArG rCrUrG rGrGrA

Table S1. siRNA guide pairs from Integrated DNA Technologies used to confirm antibody specificity. Each siRNA pair is in one column with each sequence separated within the cell.

Target	Species React	Vendor	Catalog Number	Epitope (aa)
NR1	rt	Millipore	AB9864	909-938
NR1	hu, rt, ms	Invitrogen	700685	834-938
NR1	hu, rt, ms	Cell Signaling	D65B7	660
NR2a	hu, rt, ms	Invitrogen	A6473	1253-1391

Table S2. Antibody panel against Nmdar subunits showing their vendor, species reactivity, and antibody epitope (amino acids: aa) (rt=rat, hu=human, ms=mouse). Although one antibody only had tested reactivity against rat, the protein is highly conserved across mammalia.