

Supplemental Materials and Methods

Quantification of tissue mitochondrial DNA (mtDNA) content.

Total DNA was extracted from adult mouse brain tissues of indicated genotypes using proteinase K digestion followed by a standard phenol/chloroform extraction and ethanol precipitation procedure. MtDNA content was measured by quantitative PCR on Applied Biosystems 7300 Real Time PCR System to amplify a fragment of mtDNA ND5 and normalized to the content of the invariable nuclear encoded 18S ribosomal RNA gene as previously described (1, 2) using primers of the following sequences.

ND5 primers (Ref. 1) :

ND5 forward: 5' ggcagacgaacaagacatccgaaa 3'

ND5 reverse: 5' gctaggcgtttgattgggtt 3'

r18S primers (Ref. 2) :

r18S forward: 5' acggaccagagcgaaagcat 3'

r18S reverse: 5' ggacatctaaggcatcacagac 3'

Assays for maximal enzymatic activity of each respiratory chain complex in brain mitochondria.

Purified mitochondria from adult mouse cerebrum were obtained for indicated

genotypes according to the method summarized in Fig. S1. A half of P2 pellet was suspended in 50 μ l assay buffer containing 4% digitonin and centrifuged at 20,000 g for 30 min. The supernatant was used as mitochondrial extract containing respiratory chain supercomplex. The activity of each complex was analyzed as described with slight modifications (3, 4). Briefly, Complex I activity was measured by monitoring the oxidation of NADH at 340 nm. One hundred μ l of reaction mixture consisted of 250 mM sucrose, 1 mM EDTA, 50 mM Tris-HCl (pH 7.4), 2mM KCN, 10 mM decylubiquinone (DB). 50 μ M of NADH was used to initiate the reaction. Rotenone (500 ng) was used to confirm Complex I specific activity. Complex II activity was measured by monitoring the reduction of 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm minus 520 nm, by DB in the presence of succinate. Two hundred μ l of reaction mixture consisted 50 mM KH_2PO_4 (pH 7.4), 20 mM succinate, 400 ng antimycin, 400 ng rotenone, 2 mM KCN. 50 μ M DB and 50 μ M DCPIP was added to initiate reaction. Complex III activity was measured by monitoring the reduction of cytochrome *c* at 550 nm minus 540 nm, using decylubiquinol (DBH_2) as substrate. DBH_2 was made by reduction of DB with potassium tetrahydroborate in an acidic ethanol solution. Two hundred μ l of reaction mixture contained 250 mM sucrose, 1 mM EDTA, 50 mM Tris-HCl (pH 7.4), 50 mM ferricytochrome *c*, 2 mM KCN. The reaction was initiated by addition of 50 μ M DBH_2 ,

and terminated with 1 μg antimycin A to verify complex III activity. Complex IV activity was measured by recording the oxidation ferrocytochrome *c* (fully reduced with L-ascorbate) at 550 nm minus 540 nm. Two hundred μl of reaction mixture consisted of 24 μM ferrocytochrome *c* and 10 mM KH_2PO_4 (pH 7.4). The reaction was started by addition of mitochondrial extract. Complex V activity was measured by recording the oxidation of NADH at 340 nm. One hundred μl of reaction mixture consisted of 25 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM MgCl_2 , 1.5 mM phosphoenolpyruvate (PEP), 1U lactate dehydrogenase (LDH), 0.75U pyruvate kinase (PK), 2.5 μM Rotenone, 4 mM ATP, 200 μM NADH. The reaction was started by addition of mitochondrial extract. Oligomycin (100 ng) was used to confirm Complex V specific activity. The absorbance representing enzymatic activity for each complex was measured by SpectraMax M2 (Molecular Devices) at 30°C.

References.

- (1) Casas, F. et al. (2008) PLoS ONE, 3:e2501-
- (2) Blokhin, A. et al. (2008) J. Mol. Neurosci., 35:283-287
- (3) Trounce, I. A. et al. (1996) Methods in Enzymology, 264:484-509
- (4) Bosetti, F. et al. (2000) Mol. Cell. Biochem, 215:31-38

Supplemental Figure legend

Fig. S1. Wld18 antibody specifically detects Wld^s protein in immunoblot analysis.

Lane 1: Wild-type mouse brain lysate, lane 2: *wld^s* mouse brain lysate, lane 3: Lysate of HEK293T cells overexpressing His-tagged Wld^s protein, lane 4: Control HEK293T lysate. Wld^s protein (41.5 kDa) or His-tagged Wld^s protein (42.4 kDa) was specifically detected by Wld-18. Note that Wld18 antibody and anti-His-Tag antibody recognized a protein with the same size on lane 2. Alpha-tubulin served as a loading control.

Fig. S2. Synaptic mitochondria fractionation and purification from brain tissue.

Mitochondria were purified from the mouse cerebrum by sequential centrifugation and Percoll gradient. The procedure is summarized as a flow diagram in A. Typical separation of NMNAT3-Tg cerebrum after centrifugation using the Percoll gradient is shown in B. Each 10 µl fraction was taken from the top and subjected to immunoblot analysis to examine the distribution of PSD95 (representing synaptosome), COX IV (mitochondria), and NMNAT3 (B). Layer 1 contains a considerable amount of lipids derived from myelin. Layer 2 and 3 both contained mitochondria. Layer 2 contained

synaptosome marker PSD95 and therefore is called synaptic mitochondria. On the other hand, mitochondria in layer 3 are called non-synaptic mitochondria. Layer 2 is used for subsequent functional analysis of mitochondria, because NMNAT3 is mainly expressed there.

Fig. S3. Immunohistochemical localization of transgene-derived proteins in dorsal root ganglia of NMNAT1-Tg, NMNAT3-Tg and Wld^s (W258A)-Tg mice.

Transgene-derived proteins were visualized by anti-His antibody staining (red). The same sections were co-stained by DAPI (blue) to label nuclei. Scale bar = 50 μ m.

Fig. S4. NMNAT1-Tg, NMNAT3-Tg, and Wld^s W258A-Tg mice weigh similar to wild type mice.

Average body weights of NMNAT1-Tg (n = 15), NMNAT3-Tg (n = 14) and Wld^s W258A-Tg (n = 7) mice in 57 to 72 weeks-old age were not significantly different from that of wild-type (n = 12) mice (Student t test $P > 0.05$).

Fig. S5. MtDNA content in brain tissues of *wld^s* and NMNAT3-Tg mice was similar to that of wild type mice.

MtDNA content in brain tissue was measured by quantitative PCR to amplify a fragment of mtDNA ND5. MtDNA level was normalized to the content of the invariable nuclear encoded 18S ribosomal RNA and shown as relative to the level of wild type. MtDNA content in brain tissues of *wld^s* and NMNAT3-Tg mice was not significantly different from that of wild type mice by one-way ANOVA followed by Tucky's test (n=2; $P > 0.05$).

Fig. S6. Enzymatic activity of each mitochondrial respiratory chain complex of *wld^s* and NMNAT3-Tg mice was comparable to that of wild type mice.

Enzymatic activity of each mitochondrial respiratory chain complex (Complex I through V) of *wld^s* and NMNAT3-Tg (line 822) mouse brain tissue was measured and normalized to the activity of wild type. The activities in *wld^s* or NMNAT3-Tg mice were not significantly different from those of wild type mice for all the complexes by one-way ANOVA followed by Tucky's test (n=4; $P > 0.05$).