Title: **Dysregulated brain-type creatine kinase is associated with hearing impairment in mice with Huntington's disease**

Supplementary Methods:

Participants: HD patients with middle-ear pathologies were excluded by history and physical examinations. In this study, 19 patients (11 males and eight females, aged 40–59, 49.4 ± 5.3 years old, mean ± SD) were recruited from Chang Gung Memorial Hospital (CGMH) and Taipei Veterans General Hospital (TVGH) for a complete audiologic evaluation (including PTA, speech audiometry, speech discrimination score, impedance audiometry, and ABRs) in the corresponding hospitals and Tri-Service General Hospital (TSGH). A diagnosis of HD was established by a neurological examination and genetic assessment of CAG expansion in the Htt gene. The length of the CAG repeat of HD patients was determined via polymerase chain reaction (PCR) genotyping of genomic DNA collected from leucocytes of HD patients using

primers located in the *HTT* gene (5′–Tamra-ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC–3′ and 5′–GGCGGTGGCGGCTGTTGCTGCTGCTGCTGC–3′, in CGMH; 5′–ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC–3′, 5′–GGCGGTGGCGGCTGTTGC-TGCTGCTGCTGC–3′ in TVGH). The resulting products were resolved using linear polyacrylamide gels on an automated MegaBACE analyzer (GE Healthcare). Severity and progression of HD was evaluated using the Unified Huntington's Disease Rating Scale (UHDRS) following the instruction of the Huntington Study Group (1). Three major features (i.e., motor function, independence, and functional capacity) were scored. The scale ranges (normal to most severe) for total motor score, independence scale, and total functional capacity are 0–124, 100–10, and 13–0, respectively. Thresholds of PTA and ABR waveform latencies of non-HD controls were recorded from 20 age-matched control subjects (49.3 ± 5.3 years old, mean ± SD; 10 males and 10 females) who were recruited from TSGH and had no retro-cochlear lesion based on a battery of hearing tests. The protocol was in compliance with the guidelines of the Institutional Review Boards at Academia Sinica, CGMH, TVGH, and TSGH. To compare the PTA threshold of HD patients with that of a large population of non-HD subjects, the PTA threshold of an age-matched normal population (48.5 ± 5.5 years, mean ± SD; 893 males and 977 females) was extracted from the National Health and Nutrition Examination Survey (NHANES, 1999–2004; (2)).

Hearing assessment in HD subjects: Prior to a hearing evaluation, a complete otolaryngology physical examination was performed, including otoscopy and tympanometry to exclude a conductive component of the possible hearing loss. PTAs of all subjects were obtained at octave frequencies from 250 to 8000 Hz for each ear in a sound-attenuating room using TDH-50P headphones (Telephonics) and a Grason-Stadler GSI 61 clinical audiometer (Eden Prairie, MN, USA). A descending/ascending method in 5-dB steps was used to obtain hearing threshold levels. The 4-frequency PTA of each ear was defined as the average of the PTTs at 500, 1000, 2000, and 4000 Hz. ABRs were performed with a Bio-Logic system Navigator® PRO device (Natus Medical). Stimuli of 0.1-ms clicks of alternating polarity were presented through insert earphones at a rate of 11.7/s. Recording of the latency and amplitude were made at a stimulus level of 80 dB (n)HL, with a ground electrode on the forehead and a positive electrode on the ipsilateral mastoid, and the contralateral mastoid serving as a reference. Responses were pre-amplified and band-pass-filtered at 0.1─3 kHz. Each tracing consisted of 10-ms sweeps averaged over 1024 runs.

Animals and diet administration. Male R6/2 [B6CBA-Tg(HDexon1)62Gpb/1J] (3) and littermate controls were originally obtained from Jackson Laboratories and mated to female control mice (B6CBAFI/J). In total, 75 R6/2 mice and 47 WT littermate controls were used in this study. The knock-in HD mouse model [$Hdh^{(CAG)150}$, B6.129P2- $Hdh^{tm2Detl}$/J] (4) was

originally purchased from Jackson Laboratories, and mated to C57BL/6J mice. In total, 22 homozygote $Hdh^{(CAG)150}$ mice and 22 WT littermates were used in this study. Offspring of R6/2 and $Hdh^{(CAG)150}$ were identified by the polymerase chain reaction (PCR) genotyping of genomic DNA extracted from tail tissues. Primer sequence for R6/2 were 5'-CCGCTCAGGTTCTGCTTTTA-3' and 5'-GGCTGAGGAAGCTGAGGAG-3'; and for $Hdh^{(CAG)150}$ were 5'-CCCATTCATTGCCTTGCTG-3' and 5'-GCGGCTGAGGGGGTTGA-3'. All diets used in the present study were purchased from LabDiet®. Animals were housed at the Institute of Biomedical Sciences Animal Care Facility under a 12-h light/dark cycle. Body weights of mice were recorded three times per week. Animal experiments were performed under protocols approved by the Academia Sinica Institutional Animal Care and Utilization Committee, Taiwan.

Auditory brainstem responses (ABRs) of animals. Mice auditory function was assessed by recording ABRs as described previously (5). In brief, mice were anesthetized with an intraperitoneal injection of xylazine (16 mg/kg) and ketamine (100 mg/kg) and kept warm with a heating pad in a sound-attenuating chamber. Subdermal needle electrodes were inserted at the vertex (positive), below the pinna of the ear (negative), and at the back (ground) of the mice. Specific stimuli (clicks and 4-, 8-, 16-, and 32-kHz tone bursts) were generated by the use of SigGen software (Tucker-Davis Technologies) and delivered to the external auditory canal. The

average responses from 1024 stimuli for each frequency were obtained by reducing the sound intensity in 5-dB steps until the threshold was reached. Thresholds were defined as the lowest intensity at which a reproducible deflection in the evoked response trace could be recognized.

***Rotarod performance*:** Motor coordination was assessed using a rotarod apparatus (UGO Basile) at a constant speed (12 rpm) over a period of 2min as described earlier (1). Briefly, the animals were trained for 2 days at the age of 4 weeks to allow the animals to become acquainted with the rotarod apparatus. Animals were then tested three times per week from the age of 5 weeks. Each mouse was given three trials for a maximum of 2 min for each trial. Latency to falling was automatically recorded. The best performance (i.e., the longest time spent on the rod) out of 3 trials for each animal was used for the analysis.

***Immunohistochemistry*.** Cochleae were dissected out and perfused with 4% paraformaldehyde in PBS (pH 7.4) at room temperature (RT) for 2─3 h. After fixation, cochleae were rinsed with PBS and decalcified in 5% EDTA with 0.25% glutaraldehyde until soft. Midmodiolar sections (5 μm) of decalcified cochlea embedded in paraffin used for single-antigen immunostaining were obtained and stained with the avidin–biotin–peroxidase complex (ABC) method as described previously (6). In general, we used a 1:500 dilution for the monoclonal anti-huntingtin antibody (MAB5374, Chemicon International), and nuclei were stained with methyl green or hematoxylin

(Vector Lab).

Immunofluorescence analysis of the cochlea was conducted as described previously (7). Briefly, the decalcified cochleae were immersed in rising sucrose concentrations: 2 h in 10% sucrose, 12–16 h in 15% sucrose, and 12–16 h in OCT/15% sucrose (1:1), followed by embedding in OCT. Cochlear cryosections (6 μm) were postfixed with 1.5% paraformaldehyde in PBS (pH 7.4), permeabilized with 0.01% Triton-X 100 (Sigma), and blocked in 5% normal goat serum for 2 h. Primary antibodies for CKB (1:500, clone 6F11, Developmental Studies Hybridoma Bank; Department of Biology, University of Iowa, Iowa City, U.S.), ubiquitin (1:500, DAKO, DakoCytomation, Glostrup, Denmark), and SDH-A (1:500, Abcam, Cambridge, MA, U.S.) were used as recommended by the corresponding manufacturers. The monoclonal antibody CK-JAC, developed by Glenn E. Morris, was obtained from the Developmental Studies Hybridoma Bank (developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242, U.S.).

For cochlear surface preparation, cochleae of 12-week-old WT (n = 3) and R6/2 (n = 3) mice were carefully dissected from the temporal bone and the bony shell, tectorial membrane, and lateral wall surrounding the cochlea were carefully dissected away. The remaining sensory epithelium containing the organ of Corti was fixed with 4% paraformaldehyde in PBS (pH 7.4) at RT for 30 min, followed by PBS washing, permeabilization in 0.01% Triton-X 100 (Sigma), and blocking in 5% normal goat serum for 2 h. Tissues were stained with a monoclonal

anti-huntingtin antibody (1:500 dilution, MAB5374, Chemicon International, Temecula, CA, USA) at 4 °C overnight, followed by incubation with a mixture of Alexa Fluo® 488-conjugated goat anti-mouse IgG antibody (1:250, Chemicon International) and rhodamine–phalloidin (1:200, Molecular Probes) for 2 h. Nuclei were stained with Hoechst 33258 (1 μg/ml, Sigma) at RT for 10 min. Immunolabeling was visualized with the aid of a laser-scanning confocal microscope (LSM 510 META, Carl Zeiss, Oberkochen, Germany).

Fluorescence intensity quantification. Cryosections of cochleae collected from four pairs of WT and R6/2 (12 weeks of age) and four pairs of age-matched WT and $Hdh^{(CAG)150}$ mice (15–20 months of age) were used for immunostaining and quantification. Fluorescence intensity was first analyzed using the ImageJ software (http://rsbweb.nih.gov/ij/), normalized to the corresponding area, and compared with that of WT animals (as 100%).

***Tissue collection and protein isolation*.** Animals were deeply anesthetized and decapitated. The temporal bones were rapidly removed and placed in ice-cold PBS containing the cØmplete protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland). Whole cochleae were micro-dissected from the temporal bone and stored at -80 °C until used. To obtain the total protein extract, six cochleae were pooled in ice-cold buffer (10 mM HEPES (pH 8), 1 mM DTT, 1 mM Na_3VO_4, and the cØmplete protease inhibitor cocktail), and homogenized with 20 Dounce

strokes. After centrifugation at 500 x*g* for 5 min at 4 °C, the supernatant was collected as the total protein lysate. The protein concentration was measured using the Bio-Rad protein assay reagent.

Western blot analysis. Equal amounts of protein were prepared in sample treatment buffer following the method of Laemmli (8), then the mixture was loaded and separated on 10% sodium dodecylsulfate polyacrylamide gels. After electrophoresis, the gels were transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA), blocked with 5% skim milk in TBST (0.2 M Tris-base, 1.37 M NaCl, and 0.05% Tween 20), and probed with the indicated antibody at 4 °C overnight. After being washed three times with TBST, the membranes were then incubated with a peroxidase-conjugated secondary antibody for 1 h at RT and washed another three times with TBST. The immunoreactive bands were stained using a light-emitting nonradioactive method (enhanced chemiluminescence; Millipore). Antibodies used for Western blot analyses were anti-CKB (dilution 1/5000; HPA001254, Sigma-Aldrich, St. Louis, MO, USA) and anti-actin (dilution 1/10000; MAB1501, Chemicon).

RNA purification and real-time quantitative PCR (RT-qPCR). After the mice were sacrificed, the cochleae were dissected from the temporal bone and immediately placed in ice-cold RNAlater solution (Qiagen, Valencia, CA, USA). Collected tissues were stored in liquid nitrogen

until used. Total RNA was extracted from six cochleae using TRIZOL and treated with RNase-free DNase (RQ1; Promega, Madison, WI, USA) to remove potential contamination of genomic DNA following the manufacturer's instructions. Complementary (c)DNA was synthesized from 1 μg total RNA by reverse transcription using random primers (Promega) and Superscript II RT (Life Technologies, Foster City, CA, USA) following the manufacturer's protocols. The quantitative real-time PCR (qPCR) was performed using SYBR® Green PCR Master Mix (Life Technologies) on an ABI PRISM® 7700 Sequence Detection System (Life Technologies). The sequences of primers are listed here: for *Ckb* (NM_021273), 5'-AGTTCCCTGATCTGAGCAGC-3' and 5'-GAATGGCGTCGTCCAAAGTAA-3'; and for *Gapdh* (NM_008084; used as an internal control), 5'-AGTTCCCTGATCTGAGCAGC-3' and 5'-GAATGGCGTCGTCCAAAGTAA-3'.

ATP determination of isolated hair cells. Isolation of hair cells was performed as described elsewhere with slight modifications (9, 10). Briefly, mice of 12 weeks old were decapitated. The cochleae were dissected in an artificial perilymphatic solution (APS; 125 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH2PO4, 17 mM glucose, 2 mM $CaCl_2$, 1 mM MgCl2, pH 7.3). Segments of the organ of Corti were isolated and transferred into a 50-μl droplet of APS containing collagnease type IV (1 mg/ml, Sigma) in a petri dish for 10 min at room temperature. Tissues were washed three times with fresh APS and transferred in to an 1.5-ml microtube

containing 100 μl of APS (4 cochleae per tube). The organ of Corti segments were separated by pipetting and the hair cells enriched supernatants were transferred into new tubes. Cell numbers were determined using a hematocytometer. The levels of ATP were measured by an ATP Bioluminescence Assay Kit HS II (Roche) following the manufacturer's protocols, and normalized with cell numbers. One representative experiment of three independent trials is shown.

Creatine diet supplementation. WT and R6/2 mice from the same filial generation were supplied with standard diets or diets supplemented with 2% creatine (LabDiet®, Richmond, VA, USA) from the age of 4 weeks.

Statistical analysis. Human PTA thresholds and mouse ABR thresholds were analyzed using two-way analysis of variance (ANOVA), followed by a post-hoc Bonferroni multiple-comparisons test. A *Z* test was used to compare the prevalence of hearing impairment between HD patients and the normal population. The Pearson correlation coefficient was used to investigate the correlation between clinical characteristics and hearing impairment in HD patients. A multiple linear regression was performed to analyze the relationships between hearing thresholds (4-Frequency PTA) with the age of HD patients and non-HD controls. Protein and mRNA expression levels, ATP level, immunostaining, and mitochondrial mass were

compared using a two-tailed Students' *t* test. All tests were performed using the SigmaStat software, version 3.1 (Systat Software Inc, Richmond, CA, USA). Significance was set at $P \leq 0.05$. All data are shown as mean ± SEM.

Supplementary References

1. 1996. Unified Huntington's Disease Rating Scale: reliability and consistency. Huntington Study Group. *Mov Disord* 11:136-142.

2. Bainbridge, K.E., Hoffman, H.J., and Cowie, C.C. 2008. Diabetes and hearing impairment in the United States: audiometric evidence from the National Health and Nutrition Examination Survey, 1999 to 2004. *Ann Intern Med* 149:1-10.

3. Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S.W., et al. 1996. Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* 87:493-506.

4. Lin, C.H., Tallaksen-Greene, S., Chien, W.M., Cearley, J.A., Jackson, W.S., Crouse, A.B., Ren, S., Li, X.J., Albin, R.L., and Detloff, P.J. 2001. Neurological abnormalities in a knock-in mouse model of Huntington's disease. *Hum Mol Genet* 10:137-144.

5. Wu, W.L., Wang, C.H., Huang, E.Y., and Chen, C.C. 2009. Asic3(-/-) female mice with hearing deficit affects social development of pups. *PLoS One* 4:e6508.

6. Liu, F.C., Wu, G.C., Hsieh, S.T., Lai, H.L., Wang, H.F., Wang, T.W., and Chern, Y. 1998. Expression of type VI adenylyl cyclase in the central nervous system: implication for a potential regulator of multiple signals in different neurotransmitter systems. *FEBS Lett*

436:92-98.

7. Coleman, B., Rickard, N.A., de Silva, M.G., and Shepherd, R.K. 2009. A protocol for cryoembedding the adult guinea pig cochlea for fluorescence immunohistology. *J Neurosci Methods* 176:144-151.

8. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

9. Yamashita, T., Amano, H., Ohtani, M., Harada, N., and Kumazawa, T. 1991. Isolation of and calcium kinetics in cochlear inner hair cells of the guinea pig. *Acta Otolaryngol* 111:879-884.

10. He, D.Z., Zheng, J., Edge, R., and Dallos, P. 2000. Isolation of cochlear inner hair cells. *Hear Res* 145:156-160.

Supplementary Figures

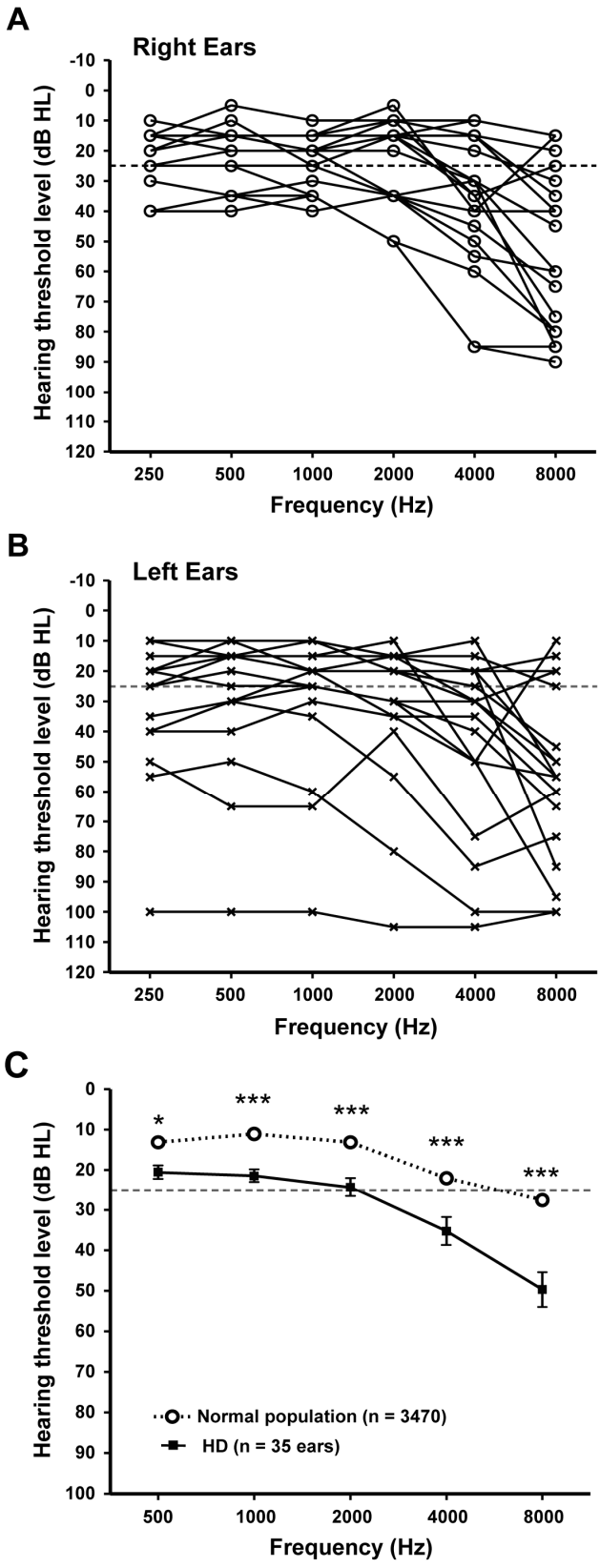

Figure S1. **Pure-tone thresholds (PTTs) of Huntington's disease (HD) patients.** (A, B) The PTTs at all octave frequencies between 250 and 8000 Hz of the right (A) and left (B) ears of HD

patients (n = 19) were plotted. (C) The mean value of the PTT at each specific frequency of HD patients (n = 35 ears) and a normal population (n = 3470 ears, data from the National Health and Nutrition Examination Survey, (2)). A dotted line indicates the normal hearing level (25 dB nHL). Data are presented as the mean ± SEM. *$P < 0.05$, ***$P < 0.001$ by two-way analysis of variance (ANOVA) followed by a post-hoc Bonferroni multiple-comparisons test. Three ears of HD patients were excluded because of middle ear pathologies.

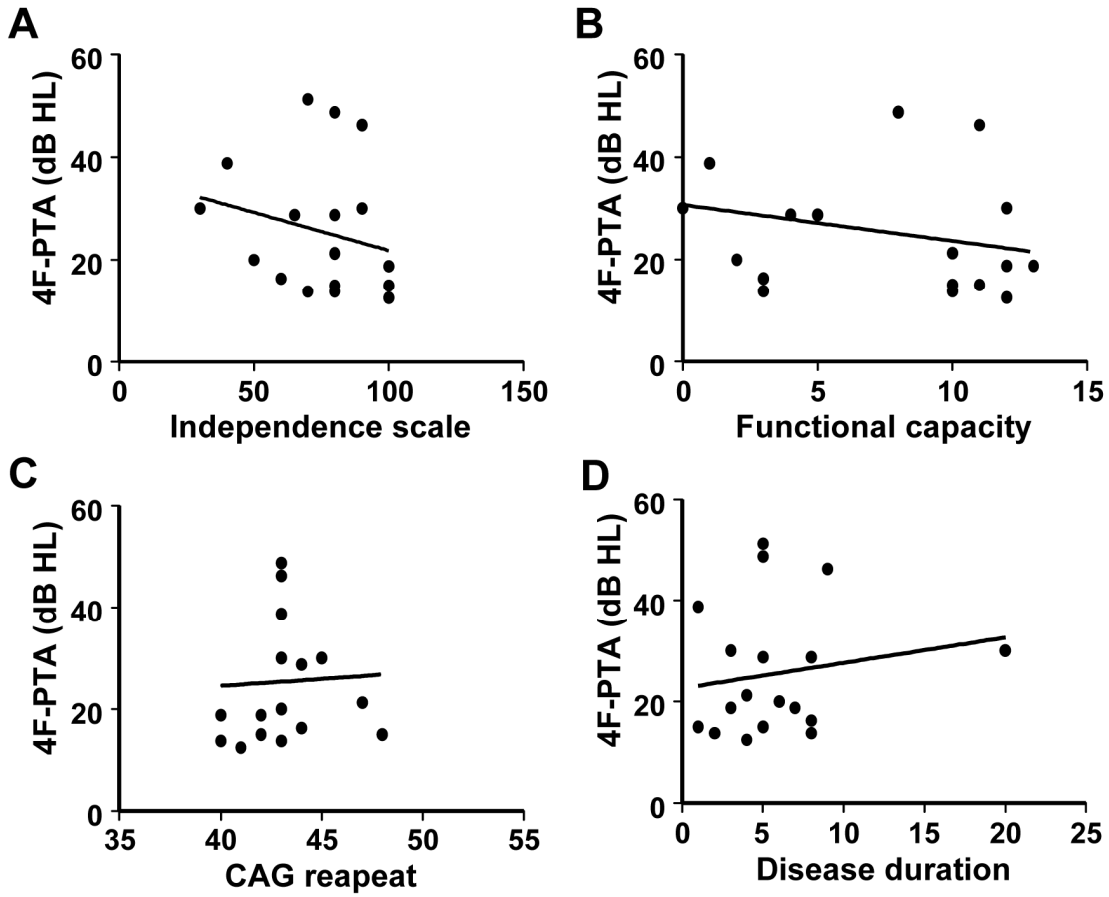

Figure S2. **Correlations between hearing impairment and clinical characteristics of HD patients.** Correlation analyses of the 4-frequency pure-tone average (4F-PTA) and independence scale scores (A, $P = 0.3283$ $r = -0.2372$), functional capacity scores (B, $P = 0.3170$ $r = -0.2426$), CAG repeat length (C, $P = 0.8570$ $r = -0.0443$), or disease duration (D, $P = 0.4852$, $r = 0.1705$) of HD patients. *$P < 0.05$, Pearson's correlation.

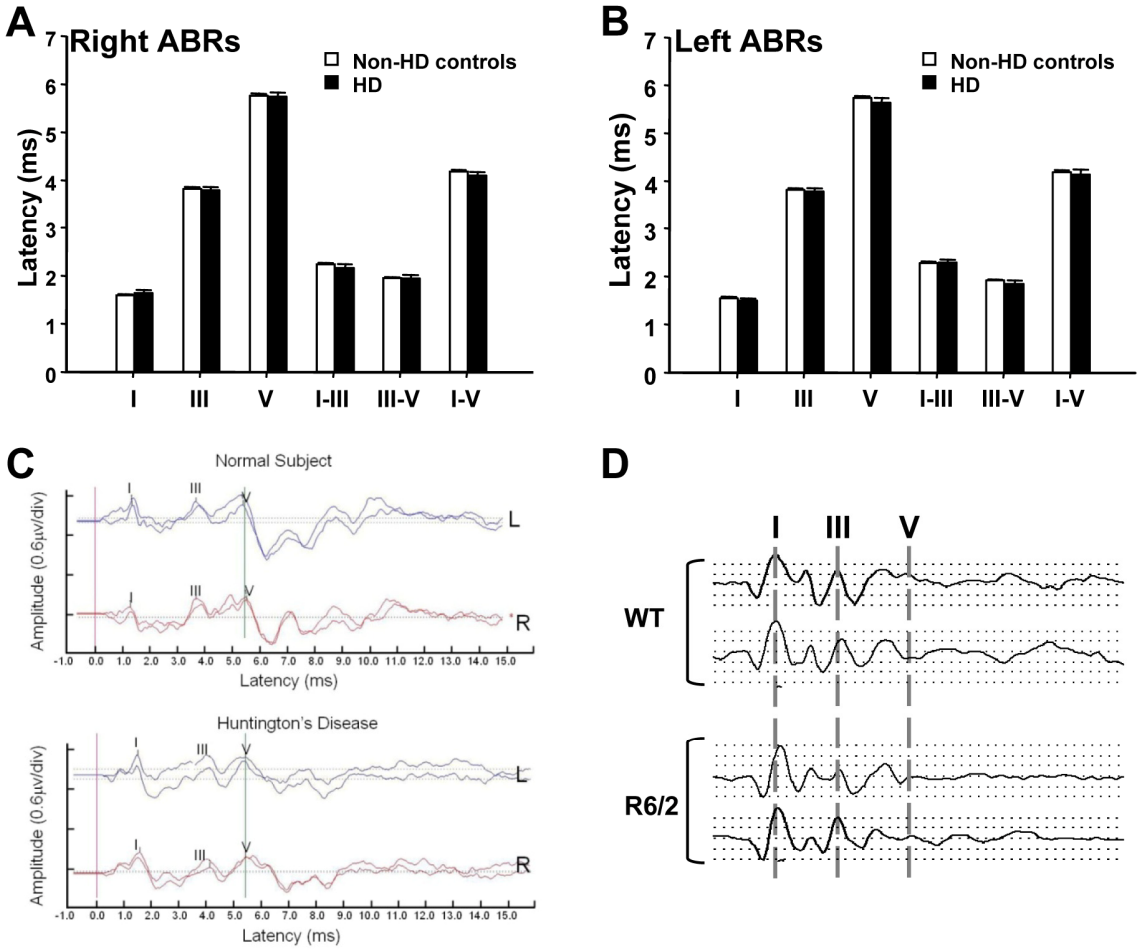

Figure S3 **Auditory brainstem response latency of HD patients and mice.** (A, B) Values (mean ± SEM, *n* = 19) of the latency of waves I, III, and V and the intervals of waves I—Ⅲ, Ⅲ—V, and I—V recorded from both the right (panel A) and left (panel B) ears of HD patients and the age-matched non-HD controls (*n* = 20). (C) ABR waveforms obtained using click stimuli at 80 dB normal hearing level (nHL) from normal subjects and HD patients are shown. Two ABR waveforms triggered by two click stimuli were obtained in each ear. L, left ear; R, right ear. (D) Representative ABR waveforms of two WT and R6/2 (10.5 weeks) at 90 dB sound pressure level (SPL) click stimulation. I, III, and V indicate the first, third, and fifth waves, respectively.

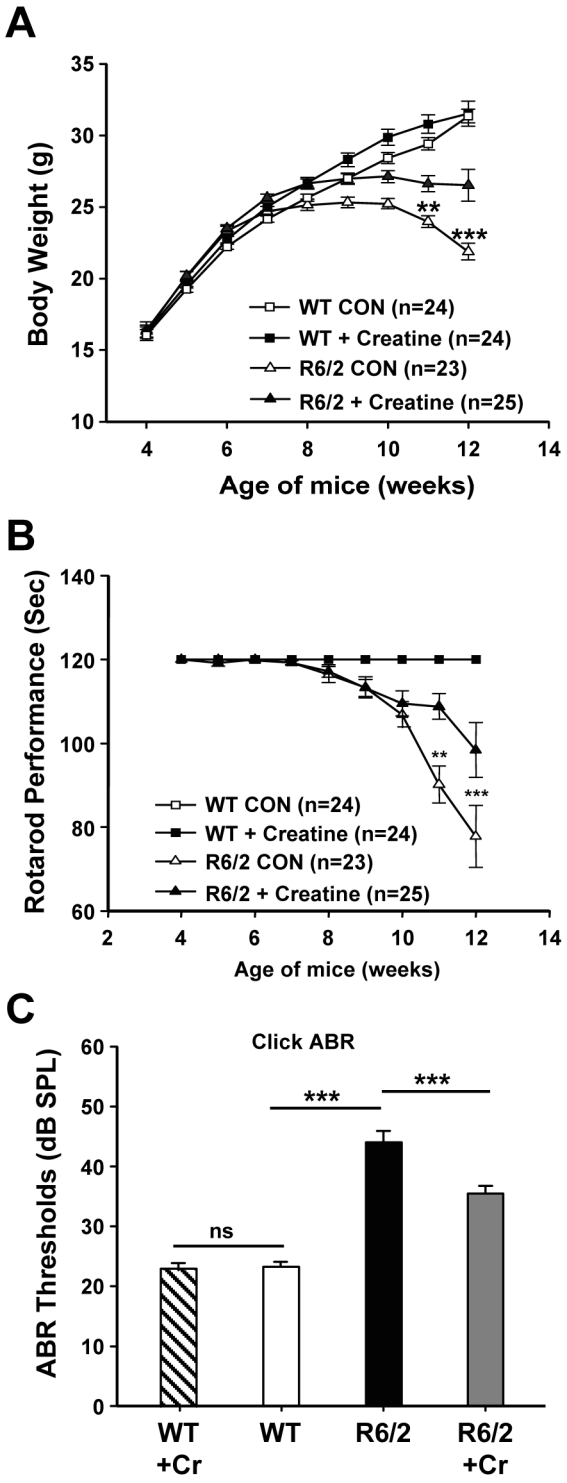

Figure S4. **Dietary creatine supplementation rescued body-weight loss and improved rotarod performance and hearing impairment of R6/2 mice.** Male mice were fed control or 2% creatine diet (Cr) from the age of four weeks. Body weight (A), rotarod performance (B), and click ABR threshold (C) were assessed. $^{**}P < 0.01$ and $^{***}P < 0.001$ and compared with R6/2 mice fed control diet at the indicated age (two-way ANOVA with Bonferroni's post-hoc tests). "ns", no significant difference. SPL, sound pressure level.

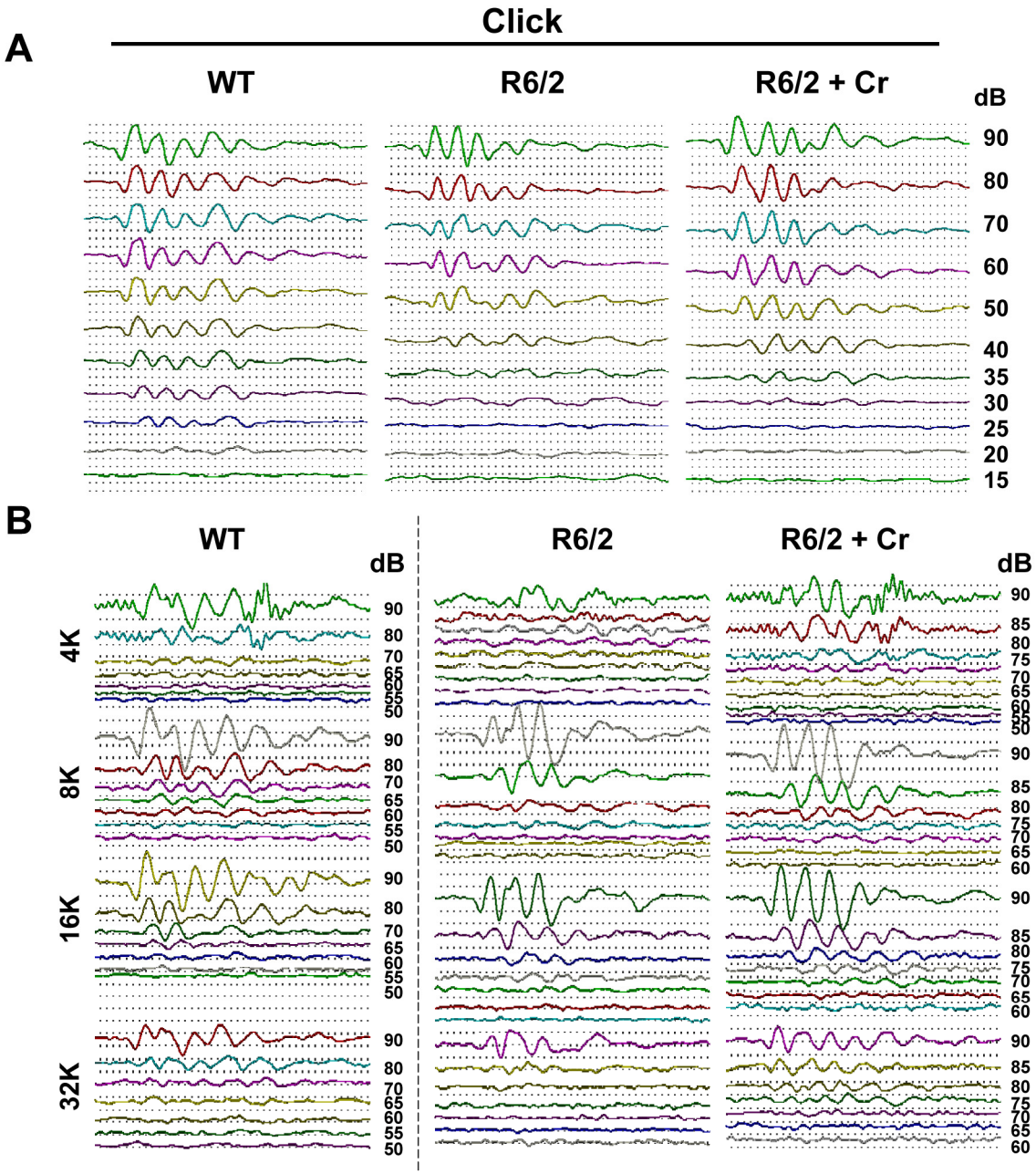

Figure S5. **Representative auditory brainstem responses (ABRs) of WT and R6/2 mice.** Male R6/2 mice were fed the control or 2% creatine diet (Cr) from the age of 4 weeks. ABR waveforms were recorded after click (A) and the indicated tone burst (B) stimuli at the age of 10.5 weeks. ABR thresholds were determined by the lowest detectable waveform.

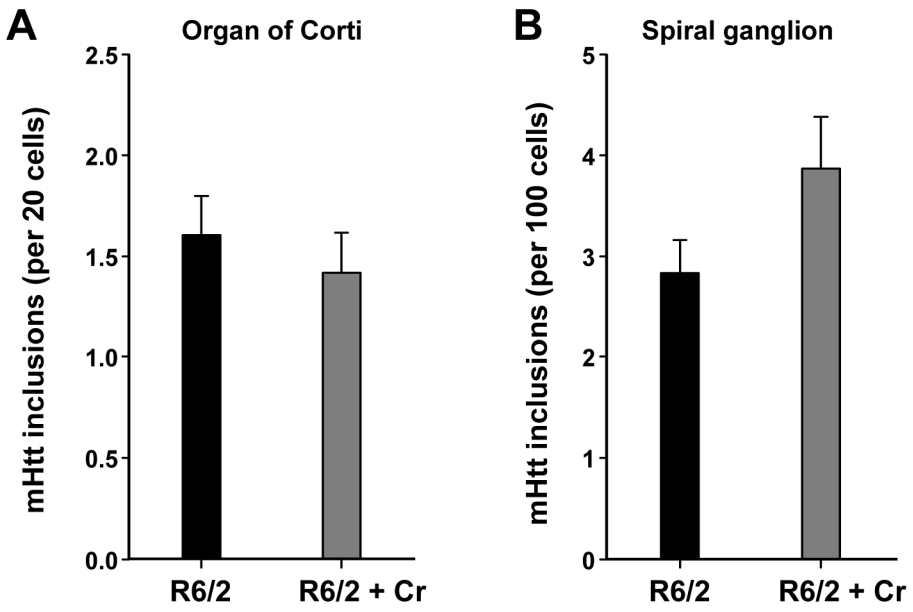

Figure S6. **Creatine supplementation did not affect the number of NIIs in the cochlea.** NIIs in the organ of Corti (A) and spiral ganglion (B) of R6/2 mice fed control diet (R6/2) and creatine supplement (R6/2 + Cr) were quantified. Cryosections of the cochleae from four animals in each group were analyzed. Data are presented as the mean ± SEM.

Table S1. **Clinical characteristics of Huntington's disease (HD) patients.** Data are presented as the mean ± SEM.

Subjects	**Non-HD controls**	**HD patients**
Mean age (years)	49.3 ± 1.2	49.4 ± 1.2
Gender	10 males and 10 females	11 males and 8 females
Mean onset age (years)		43.9 ± 1.5
Disease duration		5.5 ± 1.0
CAG Repeats		43.1 ± 0.5
Motor score		29.7 ± 3.6
Functional capacity		7.5 ± 1.0
Independence scale		76.1 ± 4.6

Table S2. **Clinical characteristics and pure-tone thresholds of HD patients.**

Subject ID	Gender	Age	onset age	duration	motor	Indep. Scale	Functional capacity	CAG Repeat	LEFT EAR						RIGHT EAR					
									250	500	1000	2000	4000	8000	250	500	1000	2000	4000	8000
C1001	M	58	53	5	56	70	5	42	40	30	35	55	85	75	30	35	35	50	85	85
C1002	M	48	45	3	10	100	13	42	15	15	15	10	50	10	20	15	15	5	40	15
C1003	F	53	48	5	31	100	11	42	25	15	20	20	20	20	25	20	20	10	10	15
C1005	M	45	41	4	24	80	10	47	15	15	20	20	30	55	15	20	20	20	30	85
C1008	F	45	41	4	22	100	12	41	15	15	10	15	10	55	10	15	15	15	30	60
C1009*	F	48	42	6	26	50	2	43	50	65	65	40	75	60	25	25	25	15	15	20
C1010	F	46	45	1	20	80	10	42	20	15	10	20	20	50	20	15	15	15	15	35
C1011*	M	58	53	5	38	80	8	43	55	50	60	80	>100	>100	25	25	35	50	85	90
C1012	M	58	51	7	23	100	12	40	15	15	15	15	30	20	15	15	15	15	35	25
C1013	F	41	39	2	24	80	10	43	10	15	15	15	15	25	15	15	15	10	15	40
C1014*	M	47	38	9	39	90	11	43	>100	>100	>100	>100	>100	>100	40	40	35	50	60	80
2001	M	41	40	1	15	80	11	48	10	10	20	15	20	15	15	15	20	15	10	15
2003	M	48	47	1	21	40	1	43	40	40	30	35	50	55	40	35	40	35	55	60
2007	M	55	52	3	21	90	12	43	20	25	25	30	50	95	20	15	20	35	50	80
2008	M	48	40	8	4	70	3	40	20	10	10	15	20	85	15	5	10	10	35	75
2009	M	50	45	5	29	80	4	44	25	20	25	30	40	65	20	10	25	35	45	65
2011	F	51	43	8	65	65	5	44	40	30	25	30	30	50	40	35	30	35	30	45
2012	F	46	26	20	54	30	0	45	35	30	20	35	35	60	30	35	30	35	40	40
2016	F	53	45	8	42	60	3	44	10	15	20	20	25	45	10	15	15	15	20	30
mean	11M	49.4	43.9	5.5	29.7	76.1	7.5	43.1	25.6	23.9	24.4	27.8	35.6	49.4	22.6	21.3	22.9	24.7	37.1	50.5
S.D	8F	5.3	6.4	4.3	15.9	20.2	4.4	2.0	14.2	14.9	15.4	17.3	20.8	24.6	9.6	10.1	8.9	15.0	22.4	26.8
S.E.M		1.2	1.5	1.0	3.6	4.6	1.0	0.5	3.4	3.5	3.6	4.1	5.0	6.0	2.2	2.3	2.0	3.5	5.1	6.2
median		48.0	45.0	5.0	24.0	80.0	10.0	43.0	20.0	15.0	20.0	20.0	30.0	55.0	20.0	15.0	20.0	15.0	35.0	45.0

* Three ears of HD patients were excluded because of middle ear pathologies.

Table S3**. Prevalence of hearing impairment (pure-tone thresholds (PTTs) ≥ 25 dB HL) in patients with Huntington's disease (HD) and an age-matched group of normal subjects recruited in the National Health and Nutrition Examination Survey (NHANES)** (2)

	NHANES controls		HD patients		
Frequency	prevalence	95% CI.	prevalence	95% CI.	*p* value
500 Hz	6.5%	5.5-7.7	36.8%	16.3-61.6	0.0001***
1000 Hz	5%	4.0-6.0	42.1%	20.3-66.5	< 0.0001***
2000 Hz	9.3%	8.0-10.7	42.1%	20.3-66.5	0.0002***
4000 Hz	27.5%	25.5-29.7	57.9%	33.5-79.8	0.0075**
8000 Hz	36.9%	34.7-39.1	78.9%	54.4-94.0	0.0004***
4F-PTA	8.2%	7.0-9.5	42.1%	20.3-66.5	< 0.0001 ***

4F-PTA, the 4-frequency PTA of better ear.

Table S4. **Multiple regression analysis of hearing thresholds (4-Frequency PTA) and age between HD patients and non-HD controls.**

Model	**β**	**SE**	**t**	***P*-value***
(Constant)	-26.250	13.613	-1.298	0.062
HD/non-HD	10.299	2.833	3.636	<0.001
Age	0.837	0.269	3.109	0.004

*Significance, $P < 0.05$. β, regression coefficient; SE, standard error.

Note: 4-Frequency PTA (dB HL) was set as a dependent variable; age (years) and disease state (HD as 1 and non-HD controls as 0) were set as independent variables. Multiple regression model: 4F-PTA = –26.250 + (10.299 × HD/non-HD) + (0.837 × age) ($P < 0.001$).