Supporting Information

Single-Walled Carbon Nanotubes Alleviate Autophagic/Lysosomal Defects in Primary Glia from a Mouse Model of Alzheimer's Disease

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Methods and Materials

SWNT preparation

SWNT were obtained commercially (Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences), and further prepared as the highly dispersed SWNT, which was described by our previous study.[1, 2] SWNT (0.5 mg/ml) were diluted in media (10% FBS) to achieve the final concentration (0.05 μg/ml).

Atomic Force Microscopy (AFM)

SWNT were deposited by electrostatic adsorption onto mica. Freshly cleaved mica was pretreated with 2.0 mg/ml poly(diallyldimethylammonium chloride) (PDDA) for 1 h, and then soaked in a solution containing fractionated SWNTs for 30 min. This substrate was rinsed briefly with water and blow-dried with air. AFM measurements were performed on a Dimension 3100 (Digital Instruments, California, CA) in tapping mode at room temperature (25-27 °C) and 20-30% humidity.

High Resolution Transmission Electron Microscopy (HR-TEM)

SWNTs were suspended in deionized water purified in a Milli-Q system (Millipore Corporation, Billerica, MA), and deposited on the mesh grids. Samples were analyzed with a Tecnai G2 F20 UT transmission electron microscope (FEI, Hillsboro, OR) at 200 kV.

Animals and primary culture

All animal procedures were performed following the National Institutes of Health Guidelines for the Humane Treatment of Animals, with approval from the Institutional Animal Care and Use Committee (IACUC) at the Nathan Kline Institute for Psychiatric Research. CRND8 mice, which express mutant human APP, Swedish (K670N/M671L) plus Indiana (V717F) mutations, were created on a 129S6/SvEvTac (129S6) strain background by Dr. David Westaway at the University of Toronto.[3]

Primary glial cells were derived from E17 pregnant CRND8 mice. Pup brains were collected separately and placed in ice-cold Hibernate-E (HE)

medium (BrainBits LLC) and the cerebral cortices were dissected. After mincing with a scalpel, cells were dissociated in HE medium containing 15 U/ml papain enzyme (Worthington Biochemicals, NJ) and DNase (0.2 μg/ml, Life Technologies) for 15 min at 37 °C. Samples were centrifuged at 1000 rpm for 5 min at room temperature. After removing the supernate, the cell pellet was resuspended with 10 ml medium and triturated for 10 times. Undissociated brain tissue was removed by filtering the cell suspension through a 40 μm nylon cell strainer (Thermo Fisher Scientific). After counting the cells, the cell suspension was diluted with DF12 complete medium (DMEM/F12 medium (Life Technologies, Carlsbad, CA, USA; 11 995-073) supplemented with penicillin/streptomycin (Life Technologies), 5% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 5% heat-inactivated horse serum) at 0.5 × 10^6 cells/ml. Primary glial cells were grown in 35 mm dishes coated with poly-D-lysine (100 μg/ml, Sigma) for 14 days at 37 °C and 5% CO_2. One-half of the plating medium was exchanged every 3 days.

Self-Clearance system

The peristaltic pump (BT300-2J, Longer Pump Precision Pump Co., Ltd, China) run at a constant speed (100 rpm) using PCV Orange-yellow manifold tubing (I.D. 0.020 in, Fisher Scientific, IL) to yield a supply constant speed (100 rpm) of media overflowing cells as.depicted in Figure 1A and Figure S3. The system supplied cultured media with SWNT (0.05 μg/ml) for 10 h, then changed the fresh medium for another 14 h.

Cytotoxicity of SWNT

Cell viability was determined using the CytoTox 96 Cytotoxicity Assay (Promega, Madison, WI) as per manufacturer's instructions. Cells were plated at 0.5x10^4/well in 100 μl DF12 medium as described above on poly-D-Lys coated plates. Standard curves were constructed using cells plated and cells plated in triplicate were assayed for viability for each condition following culture for 24 h. Results were expressed as the average ± S.D. following

spectrophotometry at 490 nm as recommended by the manufacturer.

Antibodies and agents for immunofluorescence labeling and western blot

The following antibodies were used in this study: RU2, rabbit polyclonal antibody directed against mouse cathepsin-D (made in-house, diluted 1:5000 for immunofluorescence and 1:10000 for western blotting). LC3 rabbit polyclonal antibody (Novus Biologicals, NB100-2200, diluted 1:1000 for western blot and 1:400 for immunocytofluorescence); glial fibrillary acidic protein (GFAP) mouse monoclonal antibody directed against pig glial fibrillary acidic protein (Sigma, G3893, diluted 1:400 for immunofluorescence and 1:1000 for western blot); Lamp2 mouse antibody (ABL-93, diluted 1:200 for immunofluorescence and 1:500 for western blot); p62 antibody (GP62-C, Progen Biotechnik, diluted 1:1000 for western blot) and Tubulin-β-III (Tuj-1, T8578, Sigma, diluted 1:1000 for western blot). Total mTOR (#2972) and Phospho-mTOR (#2971, pSer2448), total ULK1 (#8054) and Phospho-ULK1 (#6888, pSer757), total p70S6K (#9202) and Phospho-p70S6K (#9205, pThr389), and β-actin (A2066) were purchased from Cell Signaling Technologies and diluted 1:1000 for western blot.

Gel electrophoresis and immunoblotting

After treatments, primary glial Cells for Western blot analysis were washed with PBS for 3 times, and lysed in buffer containing (in mM) Tris-HCl (pH = 7.4), 50; NaCl, 150; EDTA, 1; and EGTA, 1; with 1% Triton X-100 and 0.5% Tween-20 with protease and phosphatase inhibitors. Samples were mixed with 4 × SDS sample buffer and incubated for 10 min at 90 °C. Following electrophoresis on a 4–20% Tris–glycine gradient gel (Life Technologies), proteins were transferred onto 0.45 μm PVDF membranes (Millipore) for detection of all other proteins then incubated overnight in primary antibody. HRP-conjugated secondary antibody was added the following morning and incubated for 1 h at room temperature. The blot was developed using the Novex ECL-kit (Life Technologies).

Confocal laser scanning microscopy

Glial cells were grown on glass-bottomed dishes to 70% confluency. For assessing CatD activation, live glial cells were labeled by LysoTracker DND-99 dye (1:5000, Life Technologies) and Bodipy-FL-pepstatin A (1:500, Life Technologies) for 20 min. Cells were washed to remove unbound dye then chased in dye-free media 3 times prior to imaging. To analyze lysosomal morphology, glial cells were washed with HBSS and fixed with 4% paraformaldehyde at room temperature for 20 min and then probed with CatD and Lamp2 antibodies. Alexa-Fluoro 488 and Alexa-Fluoro 568 conjugated secondary antibodies (1:500) were incubated with cells for 1 h at room temperature then washed with PBS. Cells were imaged using the laser scanning confocal microscope (LSM 510 META) with image capture using LSM software v3.5 (Carl Zeiss MicroImaging Inc.). Images were analyzed using the Image J program (NIH).

Analytical procedures

The quantitative colocalisation analysis was performed using Image J software (NIH Image). Statistical analysis was calculated by One-way ANOVA with Bonferroni's multiple comparison test using GraphPad InStat (GraphPad Software Inc.).

(1) Wang, L.; Zhang, L.; Xue, X.; Ge, G.; Liang, X. *Nanoscale* **2012,** 4, (13), 3983-3989.

(2) Wang, L. R.; Xue, X.; Hu, X. M.; Wei, M. Y.; Zhang, C. Q.; Ge, G. L.; Liang, X. J. *Small* **2014,** DOI: 10.1002/smll.201303342.

(3) Chishti, M. A.; Yang, D. S.; Janus, C.; Phinney, A. L.; Horne, P.; Pearson, J.; Strome, R.; Zuker, N.; Loukides, J.; French, J., et al. *J. Biol. Chem.* **2001,** 276, (24), 21562-21570.

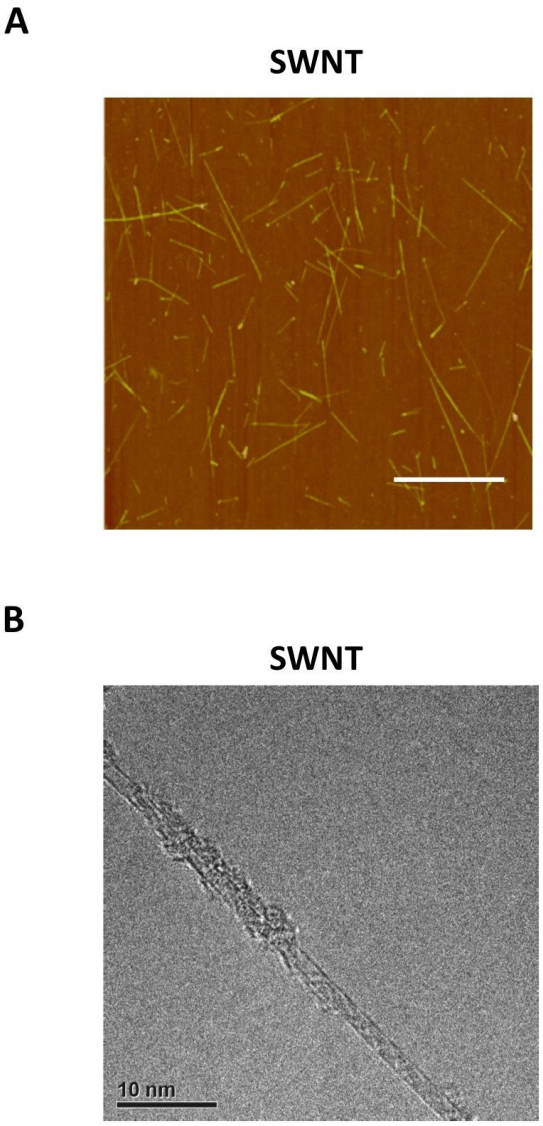

Figure S1. (A) AFM images of SWNT. Scale bar; 1 μm. (B) Typical HR-TEM micrographs of SWNT fractions. Scale bar; 10 nm.

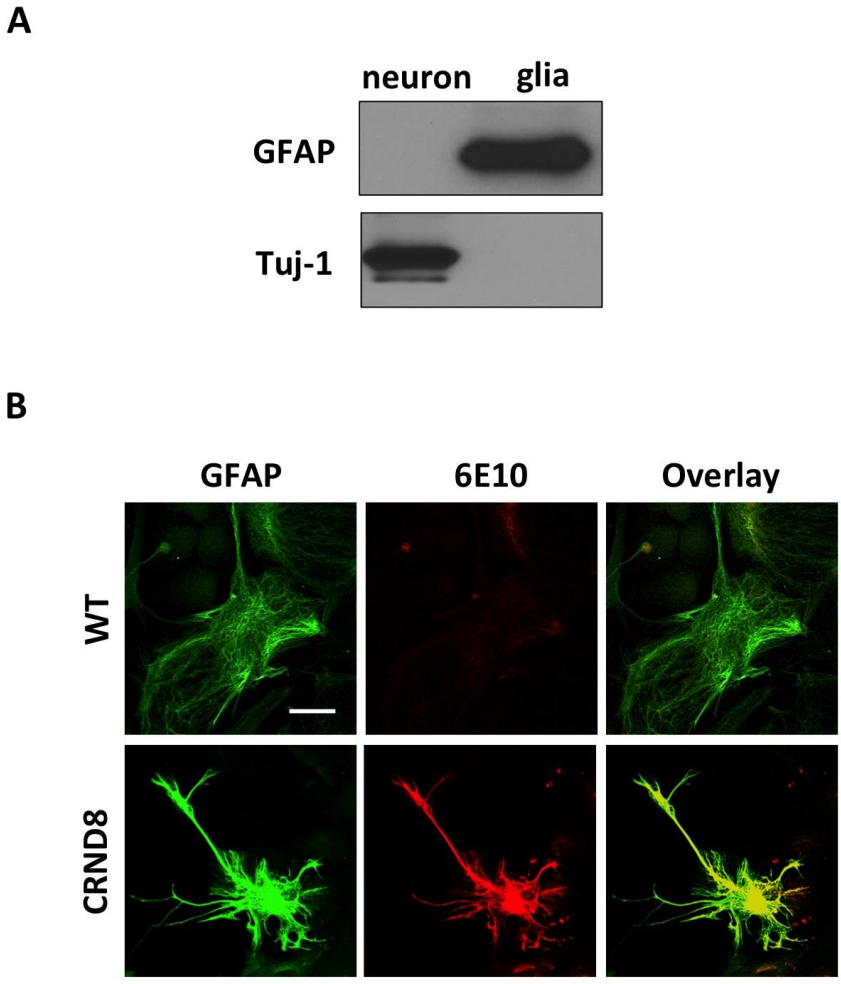

Figure S2. (A) Western blots showing the purified neuron and glia primary cultures, monitoring by glial marker GFAP and neuronal marker Tuj-1 antibodies. (B) The expression of APP in WT and CRND8 glial cells are analyzed by immunofluorescence analysis of glial GFAP (green) and Aβ-specific 6E10 antibody (red).

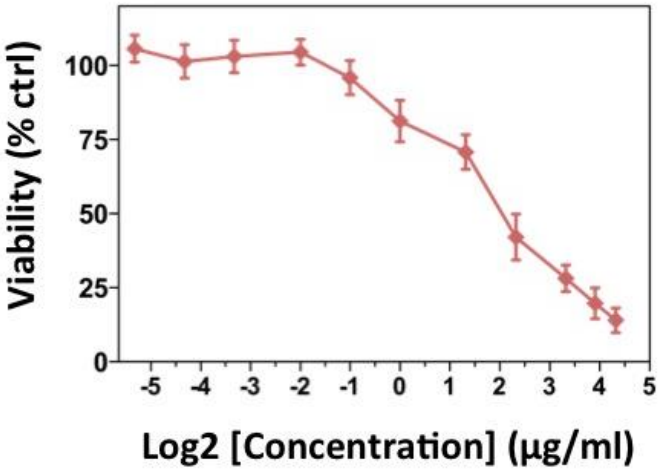

Figure S3. MTT assay indicating the glial viability against increasing concentration of SWNT following culture for 24 h.

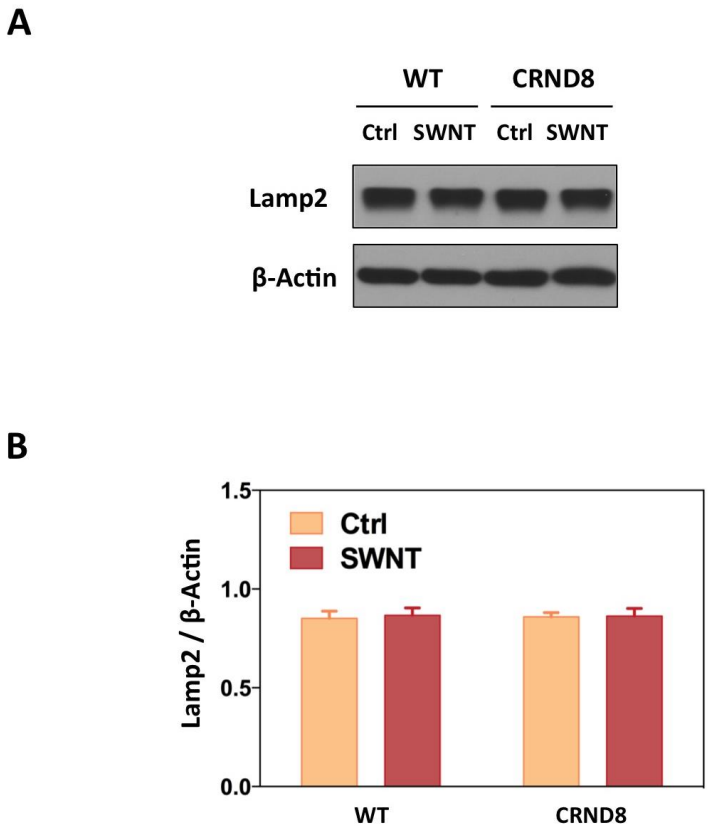

Figure S4. Representative western blot analysis of lysosomal expression with Lamp2 antibody in WT/CRND8 glial cells, showing no significant change after SWNT treatments.