**Supplementary data**

**Gintonin attenuates D-galactose-induced hippocampal senescence by improving hippocampal long-term potentiation, neurogenesis, and cognitive functions**

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**Supplementary Methods**

**Methods**

**Hippocampal neural progenitor cell culture**

Hippocampal neural progenitor cell (NPC) cultures were prepared according to the procedure described by Kim et al., (2011).Briefly, embryos at embryonic day 14.5 (E14.5) were dissected out ofC57BL/6 adult pregnant female mice. The hippocampal regionof the embryonic brain was isolated in calcium/magnesium free[Hanks’ Balanced Salt Solution](https://en.wikipedia.org/wiki/Hanks%27_salts)(HBSS). Cells were plated at 2.5 × 104 cells/cm2 on 10-cm-diameterplates coated with 15 μg/ml poly-L-ornithine and 1 μg/mlfibronectin (Invitrogen). Cells were placed in N2 medium supplementedwith B27 (Invitrogen) at 37 °C in a 95% air/5% CO2 gasincubator. Basic fibroblast growth factor (bFGF, 20 ng/ml; R&DSystems, Minneapolis, MN, USA) and epidermal growth factor (EGF, 20 ng/ml; R&D Systems) were addeddaily in order to promote proliferation of the hippocampal NPC population, andthe medium was changed every other day. Cells were sub-cultured at 80% confluencyand maintained at 6 × 104 cells/cm2 in B27-supplementedN2 medium containing bFGF and EGF.Differentiation of these sub-cultured cells was induced *via* withdrawal of bFGF and EGF, and they were maintained in the differentiationmedium (Neurobasal medium supplemented with B27) for 3–5days.

**Measurement of cell survival rate**

To measure cell survival rate, cellswere plated in 96-well plates (20,000 cells/well) overnight, and then treated with D-galactose for 24 h. The cell viability for each treatment was determined using water-soluble tetrazolium salt-1 (WST-1). The WST-1 cell proliferation assay (catalog no.: ab155902; Abcam, Cambridge, UK) was performed and the results were analyzed on a plate reader (Synergy 2, BioTek). Cells grown in a 96-well tissue culture plate were incubated with the WST-1 reagent for 0.5–4 h. After this incubation period, the formazan dye formed was quantitated using a scanning multi-well spectrophotometer (ELISA reader). The measured absorbance directly correlates to the number of viable cells.The optical density of each well was measured by a plate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA) with a filter setting at 570 nm (reference filter setting was 630 nm).

**Immunoblotting assay**

To confirm the effects of low and high concentrations of gintonin on the weight of the hippocampus as well as the expression level of doublecortin (DCX), and lysophosphatidic acid (LPA1) receptor in the hippocampus were assessed; five mice per group were sacrificed by decapitation after urethane (2 g/kg) anesthesia. The mouse brains were quickly removed, and the hippocampus was dissected free with a surgical blade and stored at -80 °C for further analysis. For immunoblotting, the brain tissues were homogenized in 50 mM phosphate-buffered saline (PBS, pH 7.4) containing 0.1 mM ethyleneglycol bis (2-aminoethyl ether)-*N*,*N*,*N′*,*N′* tetraacetic acid (EGTA, pH 8.0), 0.2% nonidet P-40, 10 mM ethylendiamine tetraacetic acid (EDTA, pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol (DTT). After centrifugation, protein concentration in the supernatants was determined using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, USA). Aliquots containing 80 μg proteins were denatured in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% sodium dodecyl sulfate, 0.3% bromophenol blue, and 30% glycerol. The aliquots were then loaded onto 10% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose membranes (Pall Corp., East Hills, NY, USA). To reduce background staining, the membranes were incubated with 5% skim milk in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween 20 for 1 h, followed by incubation with primary antibody against DCX (mouse, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or LPA1 receptor (rabbit, 1:1,000; Abcam) overnight at 4 °C. The blots were washed three times in TBS containing 0.1% Tween-20 and then incubated with horseradish peroxidase-conjugated secondary antibodies (mouse or rabbit, 1:2000). Bands were visualized using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA). The blot was densitometrically scanned for the quantification of relative optical density of each band using National Institutes of Health Image 1.59 software. The data were normalized against β-actin expression levels.

**Measurement of reactive oxygen species generation**

Cells were plated in 96-well plates (20,000 cells/well) overnight. The assay for reactive oxygen species (ROS) was performed as described previously (Garg and Chang, 2004). The medium from each well was removed, and the cells in each well were treated with 10 AM H2DCF-DA (100 Al/well prepared in HBSS) for 20 min at 37 °C. After removal of H2DCF-DA, cells were treated with various concentrations of D-galactose, as indicated in Figures S5–S7. The fluorescence of each well was detected by a fluorescence plate reader (Spectra Max Gemini XS, Molecular Devices) with the following wavelength settings: excitation, 485 nm; emission, 535 nm; and cutoff, 530 nm. A well containing cells without H2DCF-DA treatment was used as ‘‘blank’’ in each experiment. The reading of blank was subtracted before the results were plotted, expressed as net fluorescence units (FU).

**Supplementary Figure Legends**

**Supplementary Figure S1.** Experimental design (A). Physiological data (B) for the control, D-galactose (D-gal), gintonin (50 mg/kg)-fed D-gal, and gintonin (100 mg/kg)-fed D-gal groups. Physiological data include the change of body weight (*n* = 23–25 per group), and weight of the whole brain and hippocampus (*n* = 5 per group) during the study. \*p< 0.05, compared with the control group; \*\*p< 0.05, compared with the D-gal group. Data are presented as means ± standard error of the mean.

**Supplementary Figure S2.** Immunohistochemistry for Ki67 in the dentate gyrus of the mice in the control (A), D-galactose (D-gal) (B), gintonin (50 mg/kg)-fed D-gal (C), and gintonin (100 mg/kg)-fed D-gal (D) groups. The Ki67-positive nuclei were observed in the subgranular zone of the dentate gyrus (arrows). Note that the number of Ki67-positive nuclei was significantly decreased in the D-gal group. Both doses of gintonin increased cell proliferation, with the increase being statistically significant only in the gintonin (100 mg/kg)-fed D-gal group. GCL, granule cell layer; ML, molecular layer; PL, polymorphic layer. Scale bar = 50 μm. E: The number of Ki67-positive nuclei are expressed as a means ± standard error of the mean (*n* = 5 per group; \*p < 0.05, compared with the control group; \*\*p < 0.05, compared with the D-gal group; \*\*\*p < 0.05, compared with the gintonin (50 mg/kg)-fed D-gal group).

**Supplementary Figure S3.** Immunohistochemistry for pCREB in the dentate gyrus of the mice in the control (A), D-galactose (D-gal) (B), gintonin (50 mg/kg)-fed D-gal (C), and gintonin (100 mg/kg)-fed D-gal (D) groups. The pCREB-positive nuclei in the subgranular zone of the dentate gyrus (arrows) were counted. Note that number of pCREB-positive nuclei was significantly decreased in the D-gal group. In the gintonin (100 mg/kg)-fed D-gal group, the number of pCREB-positive nuclei was significantly increased compared with the D-gal and gintonin (50 mg/kg)-fed D-gal groups. pCREB, phosphorylated cyclic adenosine monophosphate response element binding protein; GCL, granule cell layer; ML, molecular layer; PL, polymorphic layer. Scale bar = 50 μm. E: The number of pCREB-positive nuclei in the dentate gyrus are expressed as mean ± standard error of the mean (*n* = 5 per group; \*p < 0.05, compared with the control group; \*\*p < 0.05, compared with the D-gal group).

**Supplementary Figure S4.** Effects of gintonin on D-galactose-induced alterations of DCX and LPA1 receptor expression levels. Western blot analysis of DCX and LPA1 receptor was performed by comparing the relative optical density of the immunoblot bands, which are demonstrated as a percentage of the value for the control group (*n* = 5 per group; \*p < 0.05, compared with the control group; \*\*p < 0.05, compared with the D-galactose [D-gal] group; \*\*\*p < 0.05, compared with the gintonin [50 mg/kg]-fed D-gal). The detailed experimental design has been shown in Fig. S1A. Data are presented as mean ± standard error of the mean.

**Supplementary Figure S5.** Effects of D-galactose on the survival rate of hippocampal neural progenitor cells. (A) D-galactose reduced cell survival rate in a concentration-dependent manner. Cells were treated with the indicated concentrations of D-galactose for 24 h. (B) D-galactose (30 mM)-mediated cell death was time-dependent. Cells were treated for the indicated time periods and subjected to water-soluble tetrazolium salt-1 assay, as described in the supplementary Materials and Methods section. Data are presented as the mean ± standard error of the mean (n = 9; \*p < 0.01, compared with untreated controls).

**Supplementary Figure S6.** Effects of gintonin on the D-galactose-induced decreased cell survival rate. Hippocampal neural progenitor cells were incubated with 30 mM D-galactose with the control vehicle (Con) or various concentrations of gintonin, along with water-soluble tetrazolium salt-1 (WST-1). (A) Gintonin stimulated cell survival in a concentration-dependent manner. The cells were pretreated with the indicated concentrations of gintonin 24 h before being treated with D-galactose (30 mM), and the cell survival rate was measured, as described in the supplementary Materials and Methods section. (B) Gintonin-mediated blocking of cell death was time-dependent. Cellswere treated with 1 µg/ml gintonin and subjected to WST-1 assay, as described in the supplementary Materials and Methods section. Data are presented as mean ± standard error of the mean (n = 9; \*p < 0.01, compared with untreated controls).

**Supplementary Figure S7.** Effects of gintonin on D-galactose-induced intracellular reactive oxygen species (ROS) generation.(A) Gintonin attenuates D-galactose-induced ROS formation in a concentration-dependent manner. Cells were pretreated with the indicated concentrations of gintonin 24 h before being treated with D-galactose (30 mM), and the level of ROS generated were measured as described in the supplementary Materials and Methods section. (B) The level of ROS generated was measured after the indicated duration of gintonin (10 μg/ml) and D-galactose (30 mM) treatment. The maximal inhibitory effect of gintonin on D-galactose-induced ROS generation was observed after 24 h. \*p < 0.01, compared with control group (Con) (n = 9).

**References**

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