Short Communication

Extracellular Vesicles Derived from *Lactobacillus plantarum* Increase BDNF Expression in Cultured Hippocampal Neurons and Produce Antidepressant-like Effects in Mice

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Gut microbiota play a role in regulating mental disorders, but the mechanism by which gut microbiota regulate brain function remains unclear. Gram negative and positive gut bacteria release membrane-derived extracellular vesicles (EVs), which function in microbiota-host intercellular communication. In the present study, we investigated whether *Lactobacillus plantarum* derived EVs (*L*-EVs) could have a role in regulating neuronal function and stress-induced depressive-like behaviors. HT22 cells treated with the stress hormone glucocorticoid (GC; corticosterone) had reduced expression of *Bdnf* and *Sirt1*, whereas *L*-EV treatment reversed GC-induced decreased expression of *Bdnf* and *Sirt1*. The siRNA-mediated knockdown of *Sirt1* in HT22 cells decreased *Bdnf4*, a splicing variant of *Bdnf*, and *Creb* expression, suggesting that Sirt1 plays a role in *L*-EV-induced increase of BDNF and CREB expression. Mice exposed to restraint for 2-h daily for 14 days (CRST) exhibited depressive-like behaviors, and these CRST-treated mice had reduced expression of *Bdnf* and *Nt4/5* in the hippocampus. In contrast, *L*-EV injection prior to each restraint treatment blocked the reduced expression of *Bdnf* and *Nt4/5*, and stress-induced depressive-like behaviors. Furthermore, *L*-EV treatment in CRST-treated mice also rescued the reduced expression of *Bdnf*, and blocked stress-induced depressive-like behaviors. These results suggest that *Lactobacillus* derived EVs can change the expression of neurotropic factors in the hippocampus and afford antidepressant-like effects in mice with stress-induced depression.

Key words: Extracellular vesicles, *Lactobacillus plantarum*, BDNF, Antidepressant-like effects

INTRODUCTION

Gut microbiota have an influence on cognition and behavior [1, 2]. Recent studies have reported that patients with depression had reduced composition of *Bacteroidetes*, *Bifidobacterium* and *Lactobacillus* in the gut microbial flora [3-5]. Supplement of probiotic capsules containing *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum* for 8 weeks in depression patients produced beneficial effects on depressive symptoms [6]. Ingestion of fermented milk containing *Lactobacillus casei* diminished anxiety and physical symptoms of medical students under academic examination [7]. Studies in animal models also supported the

beneficial effects of *Lactobacillus* on brain function. Supplement of *Lactobacillus rhamnosus* in mice displaying stress-induced depressive symptoms produced antidepressant effects and reduced the level of stress-induced increased corticosterone levels [8, 9]. Ingestion of *Lactobacillus helveticus* in rats with chronic restraint stress improved anhedonia and suppressed anxiety [10]. The level of *Lactobacillus* in fecal samples of mice was correlated with stress-induced depressive-like behavior, whereas depressive-like symptoms were ameliorated by treatment with its metabolite, kynurenine [11]. Collectively, these studies consistently support the notion that various strains of *Lactobacillus* produce beneficial effects on depressive symptoms of depression patients or stress-induced depressive-like behaviors in animal models.

Several studies have attempted to explore the mechanisms of how gut microbiota influence brain function. Gut microbiota appear to maintain host homeostasis by communicating directly and indirectly with the nerve system [1, 12, 13]. Several underlying mechanisms have been proposed to explain how gut bacteria affect neural function; (i) bacterial metabolites including short chain fatty acids, carbohydrates, bile acids [14, 15], and kynurenine [11], (ii) cytokines such as IL-6, MCP-1, TNFα, and INFα which were secreted from monocytes after stimulation with gut microbiota [16-18], and (iii) bacterial neurometabolites including dopamine, GABA, tryptophan or 5-HT precursors [19-21]. These products and metabolites are believed to enter the circulatory system and affect brain function. In contrast to this view, (iv) retrograde transport of bacterial metabolites directly through the vagus nerves innervating gut epithelial cells could occur and thereby change neural function [22-24].

Recent studies demonstrated that gut bacteria release membrane-derived extracellular vesicles (EVs) [25]. EVs carry nucleic acids, lipids, proteins, and bacterial metabolites, which can affect various cellular pathways in the host [26]. For example, *Akkermansia muciniphila* derived EVs produced protective effects on LPS-induced intestinal permeability changes through increasing phosphorylation of AMPK and tight junctions [27] and attenuate colitis-induced inflammation [28]. EVs carrying capsular polysaccharide (PSA) derived from *Bacteroides fragilis* prevented colitis by enhancing immunomodulatory effects [29]. Thus, EVs derived from specific strains of bacteria could function as novel mechanisms mediating physiological effects of the microbiota on the host. However, it is unknown whether bacteria-derived EVs can directly affect brain function.

Lactobacillus plantarum is a gram-positive bacterium that is present in dairy, fermented vegetables, and the gastrointestinal tract [30, 31]. *L. plantarum* is known to function as an immunomodulator on human colon cancer cells [32]. Recent studies

have reported the beneficial protection of *L. plantarum* on stress-induced behavior dysfunction. Administration of living or dead *L. plantarum* improved cognitive deficits against aluminum-induced brain and liver injuries in mice [33] and attenuated anxiety behavior of stressed zebrafish [34]. Administration of *L. plantarum* MTCC 9510 reduced oxidative stress markers and inflammatory cytokines in the brain and serum and improved stress-induced behavioral despair [35].

In this study, we investigated whether EVs produced by *L. plantarum* could induce changes in the expression of BDNF and improve stress-induced depressive-like behaviors.

MATERIALS AND METHODS

Preparation of EVs from Lactobacillus plantarum

Bacterial culture and EV isolation were carried out as described previously [36]. *Lactobacillus plantarum* (KCTC 11401BP, GenBank accession No. GQ336971) was cultured in MRS broth (MB Cell, CA, USA) for 18 h at 37℃ with gently shaking (150 rpm). When the optical density of the culture reached 1.0 at 600 nm, bacteria were pelleted at 10,000 ×g for 20 min and the supernatant was passed through a 0.22-μm bottle-top filter (Corning, NY, USA) to remove remaining cells. The filtrate was concentrated through a MasterFlex pump system (Cole-Parmer, IL, USA) using a 100 KDa Pellicon 2 Cassette filter membrane (Merck Millipore, MA, USA) and subsequently passed through a 0.22-μm bottle-top filter (Corning, NY, USA). The extracellular vesicles were obtained by ultracentrifugation at 150,000 ×g for 3 h at 4℃. Protein concentration was measured with BCA assay (Thermo Fisher Scientific, MA, USA) and the collected EV fractions were stored at –80℃ until use. The size of purified *L*-EVs was in the range of 20~100 nm in diameter, which was described recently [36].

HT22 cell culture and L. plantarum-EVs (L-EVs) treatment

HT22 cells were cultured as described previously [37, 38]. In brief, HT22 cells were grown in Dulbecco's modified Eagle's medium (DMEM; LM001-05, Welgene, Gyeongan-si, Korea) containing 10% heat-inactivated fetal bovine serum (FBS; FB02-500, Serum Source International, Charlotte, NC, USA) and penicillin (20 units/ml)/ streptomycin (20 mg/ml) (LS020-02, Welgene) at 37℃ and 5% CO_2 supply conditions. At 70~80% confluency, HT22 cells were trypsinized and counted using a trypan blue (0.4%) staining method. They were plated at a density of 1.0×10^5 cells/well on a 6-well plate (SPL Life Science, Pocheon-si, Gyeonggi-do), or 1.0×10^6 cells on a 100-mm dish in DMEM media supplemented with 10% FBS and antibiotics. After 24 h of culture, cells were treated with glucocorticoid (GC; corticosterone, 400 ng/ml)

or *L*-EV (20 μg/ml) in DMEM media supplemented with 1% FBS for 24 h. The dose of GC and *L*-EV was chosen based on previous studies [36, 37].

Transfection of siRNA into the HT22 cells was performed as described previously [37, 39]. HT22 cells were plated at a concentration of 1.0×10^5 cells/well in DMEM supplemented with 10% FBS in a 6-well plate (SPL Life Science). After 24 h, the media was changed with DMEM containing 1% FBS and siRNA was transfected using Lipofectamine-2000 (13778-075; Invitrogen). To prepare the siRNA and Lipofectamine-2000 mixture, 20 μM siRNA (3 μl; final concentration, 50 pM) and Lipofectamine-2000 (9 μl; final concentration, 7.5 μl/well) were separately diluted in 150 μl of Opti-MEM® Medium (31985070, Gibco, Thermo Fisher Scientific, Paisley, Scotland, UK). Each mixture was diluted at 1:1 ratio, mixed slowly and then incubated for 5 min at room temperature. The siRNA and Lipofectamine-2000 mixture (250 μl/well) was carefully dripped onto HT22 cells while culturing in fresh DMEM containing 1% FBS with or without 20 μg/well of *L*-EVs. They were then incubated for 24 h. Sirt1-siRNA was purchased from Bioneer (#1452517).

Animals, restraint and L-EV treatment

Mice were treated with restraints as described previously [40, 41]. Male C57BL/6J mice (7-weeks old) were purchased from Daehan BioLink, Inc. (Eumsung, Chungbuk, Korea). Mice were housed in pairs per cage allowing an ad libitum access to water and food at a temperature (23℃) and humidity (50~60%)-controlled room under a 12-h light/dark cycle (lights on at 07:00~19:00 h). Animals were handled in accordance with the animal care guidelines of Ewha Womans University and restraint treatment procedures in this study were approved by the Ewha Womans University Animal Care and Use Committee (IACUC 15-012). To deliver restraint, mice were placed in a 50-ml polypropylene tube carrying many punched holes for ventilation and were restrained for 2-h daily for 14 days. Control mice housed in pairs were maintained in home cages without disturbance.

L-EVs were administrated in mice in the following ways. *L*-EVs at a dose of 0.1 μg/kg each diluted in saline were intraperitoneally injected into a mouse at a volume of 100 μl 30 min prior to restraint treatment and after the 14 days of stress session, the daily *L*-EV injection was continued until the behavioral tests were completed (Exp. 1). *L*-EV treatment during the post-stress period was conducted in mice that were exposed to 2h×14d restraint treatment (Exp. 2). For the post-stress period, *L*-EVs were intraperitoneally injected at a volume of 100 μl containing increasingly higher doses; 0.1 μg/kg for the first 5 days, 0.18 μg/kg for the following 2 days, and 0.27 μg/kg for the final 7 days. Imipramine hydrochlo-

ride (IMI; I0899, Sigma) at a dose of 20 mg/kg was intraperitoneally injected at a volume of 100 μl for the indicated days.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) analysis was carried out as described previously [40, 41]. Briefly, hippocampus tissue was obtained and ground using pellet pestles (Z359971, Sigma-Aldrich) in TRI-zol reagent (15596-018, Invitrogen). Harvested HT22 cells were homogenized with TRI-zol reagent. Total RNA was isolated from homogenates and eluted in RNase free water (129112, Qiagen, Hilden, Germany). Two μg of total RNA was treated with DNase I to remove genomic DNA contamination and then converted to cDNA using a reverse transcriptase system (Promega, Madison, WI, USA).

Four μl of 1/8 diluted cDNA, 10 μl of 2X iQTM SYBR Green Supermix (Bio-Rad Laboratories, Foster City, CA, USA), and 1 μl each of 5 pmol/μl forward and reverse primers were mixed to a total volume of 20 μl. The qPCR reaction was carried out using the CFX 96 Real-Time PCR System Detector (Bio-Rad Laboratories). The transcript expression levels were normalized relative to *Gapdh* and *L32* levels.

The primers used in this study were: t*Bdnf* (total form), forward 5'-TGGCTGACACTTTTGAGCAC-3' and reverse 5'-GTTTGCGGCATCCAGGTAAT-3'; *Bdnf1*, forward 5'-CCTGCATCTGTTGGGGAGAC-3' and reverse 5'-GCCTTGTCCGTGGACGTTTA-3'; *Bdnf4*, forward 5'-CAGAGCAGCTGCCTTGATGTT-3' and reverse 5'-GCCTTGTCCGTGGACGTTTA-3'; *Creb1*, forward 5'-GAGGCAGCAAGAGAATGTCG-3' and reverse 5'-CCAGTCCATTCTCCACCGTA-3'; *Hdac2*, forward 5'-GGGACAGGCTTGGTTGTTTC-3' and reverse 5'-GAGCATCAGCAATGGCAAGT-3'; *Nt3*, forward 5'-TACTACGGCAACAGAGACG-3' and reverse 5'-GTTGCCCACATAATCCTCC-3'; *Nt4/5*, forward 5'-AGCGTTGCCTAGGAATACAGC-3' and reverse 5'-GGTCATGTTGGATGGGAGGTATC-3'; *Sirt1*, forward 5'-GATCCTTCAGTGTCATGGTTC-3' and reverse 5'-ATGGCAAGTGGCTCATCA-3'; *Trkb*, forward 5'-AAGGACTTTCATCGGGAAGCTG-3' and reverse 5'-TCGCCCTCCACACAGACAC-3'; *Gapdh*, forward 5'-AGAAGGTGGTGAAGCAGGCATC-3' and reverse 5'-CGAAGGTGGAAGAGTGGGAGTTG-3'; *L32*, forward 5'-GCTGCCATCTGTTTTACGG-3' and reverse 5'-TGACTGGTGCCTGATGAACT-3'.

Western blot analysis

Western blot analysis was carried out as described previously [40]. HT22 cells were washed with 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and harvested. Harvested HT22 cells or hippocampal tissue was homogenized in homogenization buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl,

1% Nonidet P-40, 0.1% SDS, and 0.1% sodium deoxycholate) containing a protease inhibitor cocktail (Roche) by sonicating on ice using an Epishear probe sonicator, with two rounds of 15-sec pulses and 30-sec rest intervals at 40% power outlet (Active Motif). The supernatant of homogenates was obtained after centrifugation at 13,000 ×g at 4℃ for 15 min.

The amount of protein was determined by the Bradford method (500-0006, Bio-Rad Laboratories). Twenty μg of tissue or cell sample was mixed with 6X gel loading buffer and boiled for 5 min. The proteins were resolved on SDS-PAGE and transferred onto PVDF membrane using Trans-Blot® SD semi-dry Electronic transfer cell and power supply system (1703848, Bio-Rad Laboratories). The blots were incubated with 5% skim milk or 1% bovine serum albumin (BSA) in 1X TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% Tween 20) to block a non-specific binding. Blots were incubated with a primary antibody in blocking solution followed by a secondary antibody in 1X TBST. Specific bands were visualized using Enhanced ChemiLuminescence (ECL)TM Western Blot Analysis System (RPN2109, Amersham, GE Healthcare, UK). Quantification of blot images was processed using the Image-Pro-Premier 6.0 software (MediaCybernetics, Rockville, MD, USA).

The primary antibodies used were: anti-proBDNF (ANT-006; 1:1,000, Alomone Labs, Hadassah Ein Kerem, Jerusalem, Israel), BDNF (ab108319; 1:2,000, Abcam, Cambridge, UK), and anti-β-actin (sc-47778; 1:1,000, Santa Cruz). The secondary antibodies used were: anti-mouse IgG-HRP (GTX213111-01; 1:1,000, GeneTex, Irvine, CA, USA) and anti-rabbit IgG-HRP (GTX213110-01; 1:1,000, GeneTex).

Behavioral tests

The behavioral tests were carried out as described previously [40, 41]. Mice were acclimated in the testing room for at least 30 min prior to the start of each behavioral test. All behavioral tests were performed in the light cycle phase (9 a.m.~3 p.m.) and monitored with a computerized video tracking system (SMART; Panlab S.I., Barcelona, Spain) or a webcam recording system (HD Webcam C210, Logitech, Newark, CA, USA).

Sociability test

The sociability test was performed as described previously [40, 42]. Briefly, the U-shaped two-choice field was prepared by partitioning an open field (40×40 cm^2) with a wall (20-cm wide and 20-cm high). Circular grid cages (12 cm in diameter×33 cm height) were presented on each side of the U-shaped two-choice field. For habituation, a subject mouse was allowed to freely explore the U-shaped two-choice field with empty circular grid cages on each side for 5 min and returned to the home cage. After 10 min,

a social target mouse was loaded to a circular grid cage at one side and the subject mouse was placed in the center of the U-shaped two-choice field where the subject mouse was able to see both grid cages. The subject mouse was allowed to explore both fields for 10 min while the trajectory spent in the fields was recorded by a video tracking system (SMART, Panlab S.I.) The field with a circular grid cage carrying a social target mouse and the field containing an empty circular grid cage were defined as the target field and non-target field, respectively. Social target mice with same age and sex as the subject mice were prepared. Social targets had never been exposed to subject mice from the acclimation stage.

Tail suspension test (TST)

The tail suspension test (TST) was carried out as described previously [40]. Mice were suspended individually by fixing their tails with adhesive tape to the ceiling of a shelve 50 cm above the bottom floor. The subject mouse was suspended for 6 min and the cumulative immobility time was measured. Behavioral performances were recorded with a webcam recording system (HD Webcam C210, Logitech) and subsequently analyzed.

Forced swim test (FST)

The forced swim test was performed as described previously [40]. Mice were placed in a Plexiglas cylinder (15 cm in diameter×27 cm height) holding water at 24℃ with a depth of 15 cm. Mice were placed in the cylinder for 6 min and the cumulative immobility time was measured for the final 5 min. Immobility was defined as the time when a mouse was floating with all limbs motionless. The performance during the test was recorded using a webcam recording system and then analyzed.

Statistical analysis

Multiple comparisons were performed by one-way ANOVA followed by the Newman-Keuls *post hoc* test or two-way ANOVA followed by the Bonferroni *post hoc* test. All data are represented as mean±SEM, and statistical significance was accepted at the 5% level.

RESULTS

Lactobacillus plantarum-derived EVs (L-EVs) increased the expression of BDNF in HT22 cells

Administration of microbiota products in mice increased BDNF in hippocampus [13, 43]. Treatment with probiotics mixture in aged rats increased BDNF levels in the hippocampus [44]. Administration of a probiotic formulation (*Lactobacillus helveticus* and *Bifidobacterium longum* mix) in mice rescued stressed-induced

abnormal brain plasticity and reversed the reduction of neurogenesis and BDNF levels [45]. Therefore, we investigated whether treatment with *L*-EVs in neuronal cells could induce changes in the expression of BDNF and its related factors. The hippocampal cell line HT22 cells treated with glucocorticoid (GC; corticosterone, 400 ng/ml) had decreased expression of total *Bdnf* (t*Bdnf*) and the BDNF splicing variants *Bdnf1*, *Bdnf4*, and *Ngf* compared with that of untreated control HT22 cells. In contrast, *L*-EV treat-
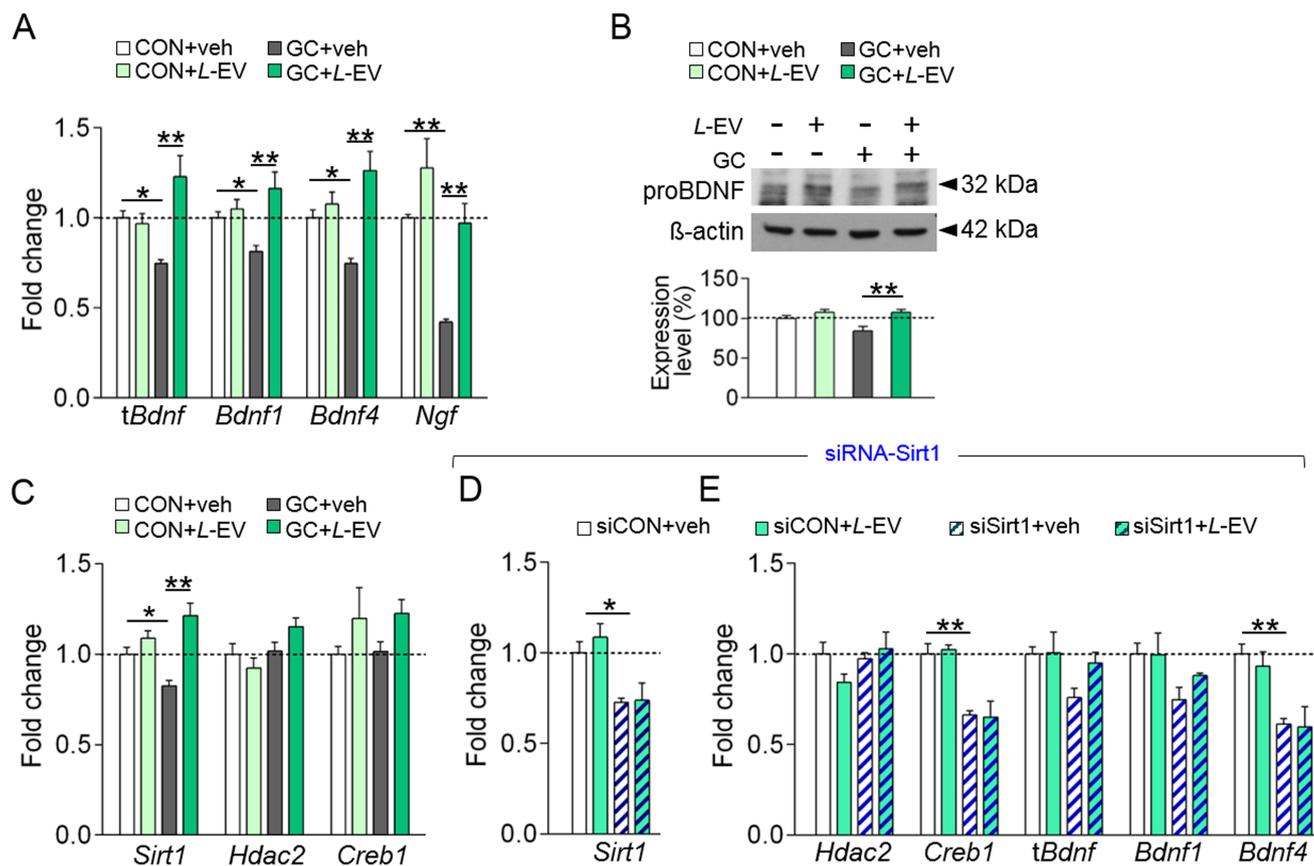
Fig. 1. *L. plantarum*-derived EVs (*L*-EVs) increased BDNF expression in HT22 cells. (A) Expression levels of t*Bdnf*, *Bdnf1*, *Bdnf4*, and *Ngf* in cultured HT22 cells in the presence of GC (400 ng/ml), *L*-EV (20 μg/ml) or GC+*L*-EV for 24 h (n=8~12 per group). Two-way ANOVA followed by Bonferroni *post hoc* test (t*Bdnf*, $F_{(1,37)}$=0.0052 and p=0.9429 for GC main effect, $F_{(1,37)}$=11.19 and p=0.0019 for *L*-EV main effect, and $F_{(1,37)}$=14.75 and p=0.0005 for GC×*L*-EV; *Bdnf1*, $F_{(1,32)}$=1.390 and p=0.2471 for GC main effect, $F_{(1,32)}$=13.60 and p=0.0008 for *L*-EV main effect, and $F_{(1,32)}$=7.166 and p=0.0116 for GC×*L*-EV; *Bdnf4*, $F_{(1,35)}$=0.2697 and p=0.6068 for GC main effect, $F_{(1,35)}$=22.60 and p<0.0001 for *L*-EV main effect, and $F_{(1,35)}$=12.49 and p=0.0012 for GC×*L*-EV; *Ngf*, $F_{(1,34)}$=27.95 and p<0.0001 for GC main effect, $F_{(1,34)}$=24.33 and p<0.0001 for *L*-EV main effect, and $F_{(1,34)}$=2.639 and p=0.1135 for GC×*L*-EV). (B) Western blot data showing the expression level of proBDNF in HT22 cells treated with GC (400 ng/ml), *L*-EV (20 μg/ml) or GC+*L*-EV for 24 h (n=6 per group). Two-way ANOVA followed by Bonferroni *post hoc* test ($F_{(1,20)}$=14.19 and p=0.0012 for GC main effect, $F_{(1,20)}$=3.819 and p=0.0648 for *L*-EV main effect, and $F_{(1,20)}$=3.819 and p=0.0648 for GC×*L*-EV). (C) Expression levels of *Sirt1*, *Hdac2*, and *Creb1* in HT22 cells treated with GC (400 ng/ml), *L*-EV (20 μg/ml) or GC+*L*-EV for 24 h (n=6~10 per group). Two-way ANOVA followed by Bonferroni *post hoc* test (*Sirt1*, $F_{(1,27)}$=0.0168 and p=0.8979 for GC main effect, $F_{(1,27)}$=65.48 and p<0.0001 for *L*-EV main effect, and $F_{(1,27)}$=22.96 and p<0.0001 for GC×*L*-EV; *Hdac2*, $F_{(1,22)}$=5.034 and p=0.0353 for GC main effect, $F_{(1,22)}$=0.2970 and p=0.5913 for *L*-EV main effect, and $F_{(1,22)}$=3.793 and p=0.0643 for GC×*L*-EV; *Creb1*, $F_{(1,26)}$=0.0513 and p=0.8227 for GC main effect, $F_{(1,26)}$=4.846 and p=0.0368 for *L*-EV main effect, and $F_{(1,26)}$=0.0056 and p=0.9412 for GC×*L*-EV). (D, E) Expression levels of *Sirt1* (D), t*Bdnf*, *Bdnf1*, *Bdnf4*, and *Creb1*(E) in HT22 cells treated with siRNA-CON (50 pmol), siRNA-*Sirt1* (50 pmol), *L*-EV (20 μg/ml) or siRNA-*Sirt1*+ *L*-EV for 24 h (n= 5~10 per group). Two-way ANOVA followed by Bonferroni *post hoc* test (*Sirt1*, $F_{(1,23)}$=22.48 and p<0.0001 for siSirt1 main effect, $F_{(1,23)}$=0.5684 and p=0.4586 for *L*-EV main effect, and $F_{(1,23)}$=0.3164 and p=0.5792 for siSirt1×*L*-EV; *Hdac2*, $F_{(1,28)}$=1.194 and p=0.2839 for siSirt1 main effect, $F_{(1,28)}$=0.4969 and p=0.4867 for *L*-EV main effect, and $F_{(1,28)}$=2.108 and p=0.1576 for siSirt1×*L*-EV; *Creb1*, $F_{(1,19)}$=47.84 and p<0.0001 for siSirt1 main effect, $F_{(1,19)}$=0.0141 and p=0.9069 for *L*-EV main effect, and $F_{(1,19)}$=0.1306 and p=0.7218 for siSirt1×*L*-EV; t*Bdnf*, $F_{(1,24)}$=3.681 and p=0.0670 for siSirt1 main effect, $F_{(1,24)}$=1.632 and p=0.2137 for *L*-EV main effect, and $F_{(1,24)}$=1.446 and p=0.2409 for siSirt1×*L*-EV; *Bdnf1*, $F_{(1,21)}$=5.607 and p=0.0276 for siSirt1 main effect, $F_{(1,21)}$=0.7048 and p=0.4106 for *L*-EV main effect, and $F_{(1,21)}$=0.7991 and p=0.3815 for siSirt1×*L*-EV; *Bdnf4*, $F_{(1,23)}$=25.64 and p<0.0001 for siSirt1 main effect, $F_{(1,23)}$=0.3326and p=0.5697 for *L*-EV main effect, and $F_{(1,23)}$=0.1315 and p=0.7202 for siSirt1×*L*-EV). GC, glucocorticoid; veh, vehicle. Data are presented as mean±SEM. *p<0.05; **p<0.01.
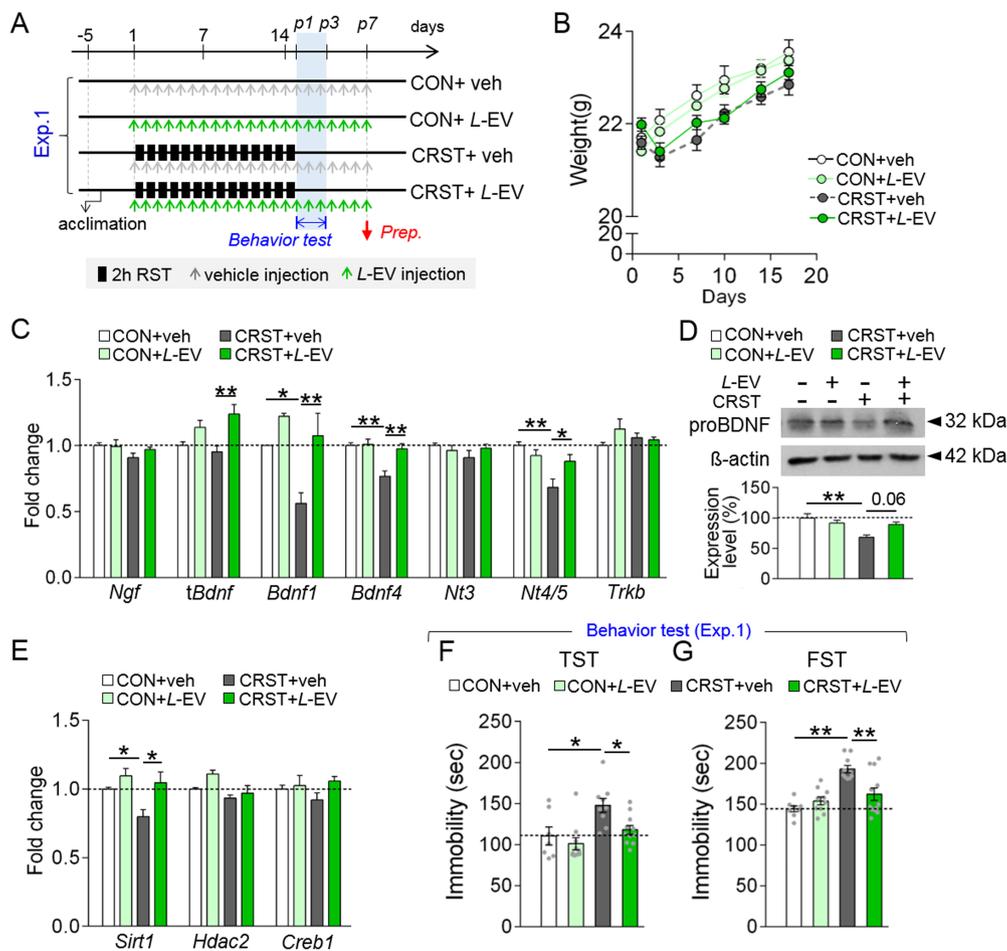
Fig. 2. *L*-EV treatment during the stress-treatment period blocked the stress-induced decrease in the expression of neurotrophic factors in the hippocampus and inhibited stress-induced depressive-like behaviors. (A) Experimental design (Exp. 1). Mice were treated with restraint for 2-h daily for 14 days (2h×14 d RST). Saline or *L*-EV (0.1 μg/kg) were i.p. injected 30 min before restraint treatment each day. Behavioral tests were performed on post-stress days 1-3 (*p1~p3*) and mice were sacrificed on post-stress day 7 (*p7*). Control mice injected with saline (CON+veh), mice treated with *L*-EV (CON+*L*-EV), mice treated with repeated restraint and injected with saline (CRST+veh), and mice treated with repeated restraint and injected with *L*-EV (CRST+*L*-EV) were prepared. (B) Body weight (g) changes of CON+veh, CON+*L*-EV, CRST+veh, and CRST+*L*-EV (n=8~12 animals per group). (C) Expression levels of *Ngf*, t*Bdnf*, *Bdnf1*, *Bdnf4*, *Nt3*, *Nt4/5*, and *Trkb* in the hippocampus of CON+veh, CON+*L*-EV, CRST+veh, and CRST+*L*-EV (n=6 animals and 4~8 PCR repeats per group). Two-way ANOVA followed by Bonferroni *post hoc* test (*Ngf*, $F_{(1,28)}$=2.900 and p=0.0997 for CRST main effect, $F_{(1,28)}$=0.6187 and p=0.4381 for *L*-EV main effect, and $F_{(1,28)}$=1.024 and p=0.3202 for CRST×*L*-EV; t*Bdnf*, $F_{(1,25)}$=0.2452 and p=0.6248 for CRST main effect, $F_{(1,25)}$=15.43 and p=0.0006 for *L*-EV main effect, and $F_{(1,25)}$=1.932 and p=0.1768 for CRST×*L*-EV; *Bdnf1*, $F_{(1,12)}$=9.542 and p=0.0094 for CRST main effect, $F_{(1,12)}$=15.01 and p=0.0022 for *L*-EV main effect, and $F_{(1,12)}$=2.324 and p=0.1533 for CRST×*L*-EV; *Bdnf4*, $F_{(1,12)}$=13.95 and p=0.0028 for CRST main effect, $F_{(1,12)}$=9.418 and p=0.0097 for *L*-EV main effect, and $F_{(1,12)}$=8.003 and p=0.0152 for CRST×*L*-EV; *Nt3*, $F_{(1,28)}$=0.9424 and p=0.3400 for CRST main effect, $F_{(1,28)}$=0.1996 and p=0.6585 for *L*-EV main effect, and $F_{(1,28)}$=1.974 and p=0.1710 for CRST×*L*-EV; *Nt4/5*, $F_{(1,24)}$=14.45 and p=0.0009 for CRST main effect, $F_{(1,24)}$=1.654 and p=0.2106 for *L*-EV main effect, and $F_{(1,24)}$=8.419 and p=0.0078 for CRST×*L*-EV; *Trkb*, $F_{(1,14)}$=0.0650 and p=0.8025 for CRST main effect, $F_{(1,14)}$=1.768 and p=0.2049 for *L*-EV main effect, and $F_{(1,14)}$=2.969 and p=0.1069 for CRST×*L*-EV). (D) Western blot data showing the expression level of proBDNF in the hippocampus of CON+veh, CON+*L*-EV, CRST+veh, and CRST+*L*-EV (n=6 animals and 4 WB repeats per group). Two-way ANOVA followed by Bonferroni *post hoc* test ($F_{(1,12)}$=12.29 and p=0.0043 for CRST main effect, $F_{(1,12)}$=1.697 and p=0.2172 for *L*-EV main effect, and $F_{(1,12)}$=8.952 and p=0.0112 for CRST×*L*-EV). (E) Expression levels of *Sirt1*, *Hdac2*, and *Creb1* in the hippocampus of CON+veh, CON+*L*-EV, CRST+veh, and CRST+*L*-EV (n=6 animals and 6-10 PCR repeats per group). Two-way ANOVA followed by Bonferroni *post hoc* test (*Sirt1*, $F_{(1,23)}$=6.483 and p=0.0181 for CRST main effect, $F_{(1,23)}$=12.17 and p=0.0020 for *L*-EV main effect, and $F_{(1,23)}$=2.365 and p=0.1377 for CRST×*L*-EV; *Hdac2*, $F_{(1,25)}$=10.31 and p=0.0036 for CRST main effect, $F_{(1,25)}$=5.110 and p=0.0327 for *L*-EV main effect, and $F_{(1,25)}$=1.340 and p=0.2280 for CRST×*L*-EV; *Creb1*, $F_{(1,22)}$=0.2456 and p=0.6251 for CRST main effect, $F_{(1,22)}$=2.829 and p=0.1067 for *L*-EV main effect, and $F_{(1,22)}$=1.358 and p=0.2564 for CRST×*L*-EV). (F, G) Immobility time in the tail suspension test (TST; F) and forced swim test (FST; G) of CON+veh, CON+*L*-EV, CRST+veh, and CRST+*L*-EV (n=7~12 animals per group). Two-way ANOVA followed by Bonferroni *post hoc* test (TST, $F_{(1,33)}$=11.90 and p=0.0016 for CRST main effect, $F_{(1,33)}$=6.235 and p=0.0177 for *L*-EV main effect, and $F_{(1,33)}$=1.665 and p=0.2059 for CRST×*L*-EV; FST, $F_{(1,36)}$=23.84 and p<0.0001 for CRST main effect, $F_{(1,36)}$=3.265 and p=0.0792 for *L*-EV main effect, and $F_{(1,36)}$=12.17 and p=0.0013 for CRST×*L*-EV). Data are presented as mean±SEM. *p<0.05; **p<0.01.

ment (20 μg/ml) reversed GC-induced reduced expression of t*Bdnf*, *Bdnf1*, *Bdnf4,* and *Ngf* (Fig. 1A). Western blot analysis confirmed that *L*-EV treatment blocked GC-induced reduced expression of proBDNF (Fig. 1B).

L-EV treatment in HT22 cells increased BDNF expression via Sirt1

BDNF expression is regulated by transcription and/or epigenetic factors, including cAMP response element (CRE) binding protein (CREB1) [46], histone acetyltransferase 2 (HDAC2) [47], and Sirtuin1 (Sirt1) [48]. HDAC2 negatively regulates BDNF expression in stress-induced depression models [47], whereas CREB1 and Sirt1 enhance BDNF expression [47-49].

GC treatment in HT22 cells decreased *Sirt1* expression, but not significantly *Creb1* and *Hdac2*, whereas *L*-EV treatment increased the GC-induced reduced expression of *Sirt1*, but produced no significant change in *Creb1* and *Hdac2* expression (Fig. 1C). The siRNA-mediated knockdown of *Sirt1* in HT22 cells reduced the expression of t*Bdnf*, *Bdnf1*, *Bdnf4*, and *Creb1*, and under the suppression of *Sirt1*, *L*-EV treatment did not increase the expression of *Bdnf4* and *Creb1* (Fig. 1D and 1E). These results suggest that *Sirt1* plays a role in *L*-EV-induced upregulation of *Bdnf4* and *Creb1*.

L-EV treatment in mice during the stress treatment phase blocked stress-induced depressive-like behaviors

Next, we examined whether *L*-EV treatment in mice affected BDNF expression in the brain and stress-induced depressive-like behaviors. Mice treated with restraint for 2-h daily for 14 days (CRST; 2h x 14d RST) exhibited depressive-like behaviors [37], and had reduced expression of BDNF in the hippocampus [50]. Consistent with these reports, mice treated with CRST had reduced expression of *Bdnf1*, *Bdnf4*, and *Nt4/5* in the hippocampus compared to that of control mice (Fig. 2A and 2C). Conversely, mice treated with *L*-EVs prior to each restraint (RST+*L*-EV; Fig. 2A) had increased expression of *Bdnf1*, *Bdnf4*, and *Nt4/5* compared to that of CRST mice (Fig. 2A and 2C). Western blot analysis also indicated that proBDNF levels were reduced after CRST treatment, whereas *L*-EV injection at each stress session blocked the stress-induced decreased expression of proBDNF (Fig. 2D).

The body weight of mice decreased during restraint treatment, but was not affected by *L*-EV treatment (Fig. 2B). Mice treated with CRST tended to have reduced expression of *Sirt1* in the hippocampus, whereas *L*-EV treatment during the stress session blocked the stress-induced reduced expression of *Sirt1* (Fig. 2E). In contrast, there was no change in *Hdac2* and *Creb1* expression. Behavioral tests indicated that mice treated with CRST exhibited

increased immobility in the TST and FST (Fig. 2F and 2G). In contrast, *L*-EV treatment prior to each restraint (Fig. 2A) blocked stress-induced increased immobility in the TST and FST (Fig. 2F and 2G).

These results suggest that *L*-EV treatment in mice during the stress induction phase blocks stress-induced reduced expression of BDNF in the hippocampus and stress-induced depressive-like behaviors.

L-EV treatment in CRST-treated mice rescued stress-induced depressive-like behaviors

Next, we examined whether *L*-EV treatment in the post-stress phase produced antidepressant-like effects. Mice treated with restraint for 2-h daily for 14 days had reduced expression of t*Bdnf*, *Bdnf4*, and *Nt3*, and tended to have reduced expression of *Bdnf1* and *Nt4/5* in the hippocampus compared to that of control mice on post-stress day 35 (p35) (Fig. 3A and 3C). Thus, stress-induced changes in the expression of these neurotrophic factors in the hippocampus were long-lasting. On the contrary, *L*-EV treatment in CRST-treated mice blocked the reduced expression of t*Bdnf*, *Bdnf1*, *Bdnf4*, and *Nt4/5* in the hippocampus (Fig. 3A and 3C). CRST-induced body weight changes occurred, whereas post-stress treatment with *L*-EVs or IMI produced no significant effect on body weight (Fig. 3B). Western blot analysis indicated that mice treated with CRST had reduced expression of proBDNF in the hippocampus, whereas post-stress treatment with *L*-EVs in CRST-treated mice suppressed stress-induced reduced expression of proBDNF and BDNF (Fig. 3D).

Mice treated with CRST showed reduced social interaction in the sociability test (Fig. 3E and 3F) and increased immobility time in the TST and FST (Fig. 3G and 3H). In contrast, post-stress treatment with *L*-EVs in CRST-treated mice reversed decreased sociability in the sociability test, and reduced stress-induced increased immobility in the TST and FST (Fig. 3E~H). These data indicated that antidepressant-like effects of *L*-EVs are comparable to those of imipramine (Fig. 3E~H).

Next, we examined whether antidepressant-like effects of *L*-EVs were sustained long after the termination of *L*-EV treatment (Fig. 3A). When reassessed on post-stress days 29-30, mice treated with CRST exhibited depressive-like behaviors in the sociability test (Fig. 3I and 3J) and in the TST and FST (Fig. 3K and 3L), whereas mice treated with CRST followed by *L*-EVs in the post-stress phase showed increased sociability and reduced immobility in the TST and FST, which were also comparable to those of mice treated with imipramine (Fig. 3I~L). These results suggest that *L*-EVs produce antidepressant-like effects in mice with stress-induced depression, and *L*-EV-induced antidepressant-like effects are stably
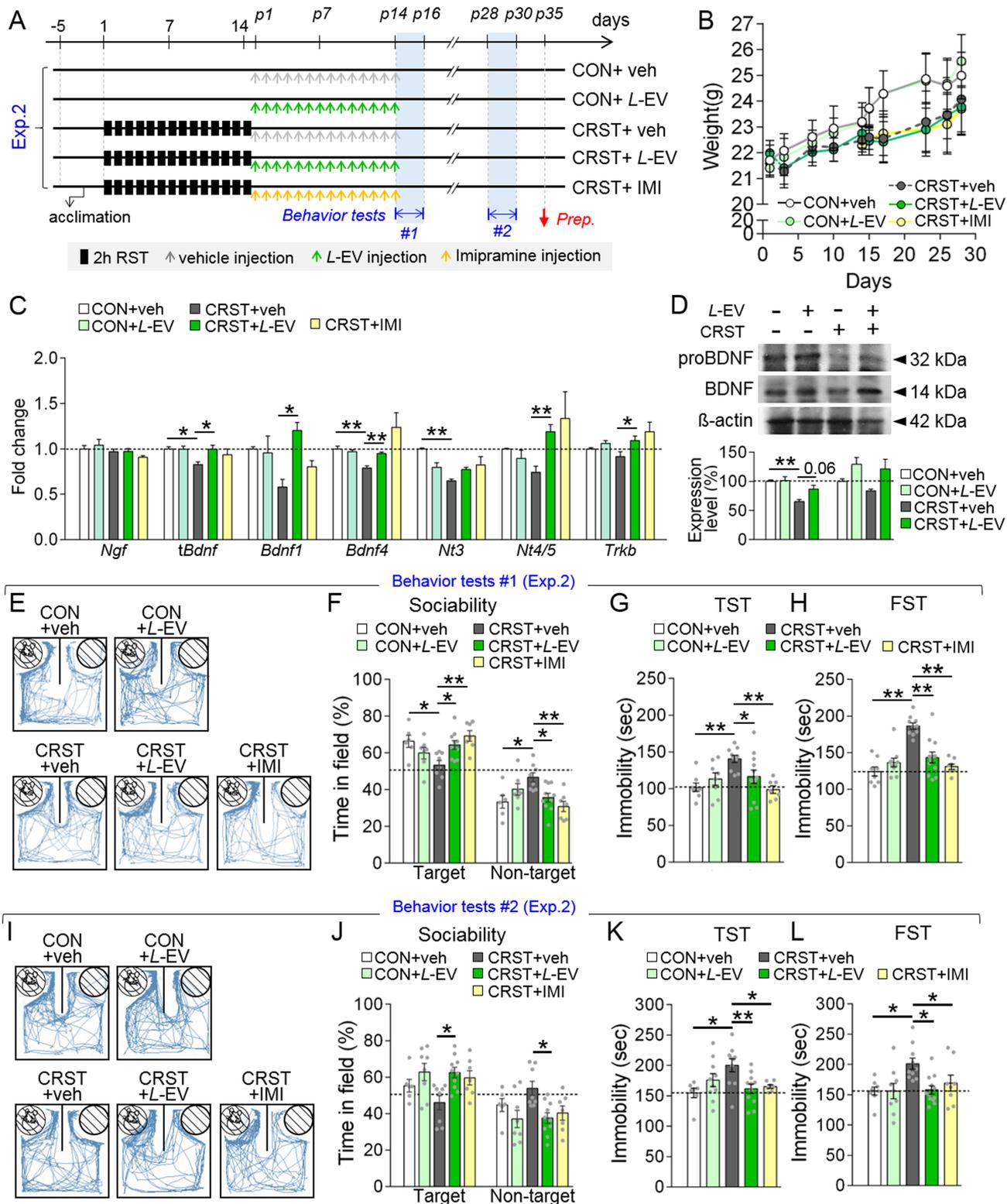
Fig. 3. *L*-EV treatment in CRST-treated mice reversed stress-induced decreased expression of BDNF in the hippocampus and rescued stress-induced depressive-like behaviors. (A) Experimental design (Exp.2). Mice exposed to CRST (2h×14 d RST) were treated with saline, imipramine (20 mg/kg), and *L*-EV (0.1 µg/kg for days 1~5; 0.18 µg/kg for days 6~7; 0.27 µg/kg for day 8 and thereafter). Behavior tests were performed on post-stress days 14~16 (*p*14~*p*16; behavior tests, #1) and post-stress days 28~30 (*p*28~*p*30; behavior tests, #2). Mice were sacrificed on post-stress day 35 (*p*35). Control mice injected with saline (CON+veh), mice treated with *L*-EV (CON+*L*-EV), mice treated with repeated restraint and injected with saline (CRST+veh),

Fig. 3. (Continued) and mice treated with repeated restraint and injected with L-EV (CRST+L-EV) and mice treated with repeated restraint and injected with imipramine (CRST+IMI) were prepared. (B) Body weight changes of CON+veh, CON+L-EV, CRST+veh, CRST+L-EV, and CRST+IMI (n=8~12 animals per group). (C) Expression levels of *Ngf*, t*Bdnf*, *Bdnf1*, *Bdnf4*, *Nt3*, *Nt4/5*, and *Trkb* in the hippocampus of CON+veh, CON+L-EV, CRST+veh, CRST+L-EV, and CRST+IMI (n=6~8 animals and 4~6 PCR repeats per group). For the comparison of CON+veh, CON+L-EV, CRST+veh, and CRST+L-EV groups, two-way ANOVA followed by Bonferroni *post hoc* test was used (*Ngf*, $F_{(1,14)}$=1.549 and p=0.2337 for CRST main effect; $F_{(1,14)}$=0.3197 and p=0.5808 for L-EV main effect; $F_{(1,14)}$=0.1826 and p=0.6757 for CRST×L-EV; t*Bdnf*, $F_{(1,14)}$=4.871 and p=0.0445 for CRST main effect; $F_{(1,14)}$=4.274 and p=0.0577 for L-EV main effect; $F_{(1,14)}$=4.752 and p=0.0468 for CRST×L-EV; *Bdnf1*, $F_{(1,12)}$=0.5907 and p=0.4570 for CRST main effect; $F_{(1,12)}$=6.682 and p=0.0239 for L-EV main effect; $F_{(1,12)}$=8.992 and p=0.0111 for CRST×L-EV; *Bdnf4*, $F_{(1,13)}$=25.35 and p=0.0002 for CRST main effect; $F_{(1,13)}$=7.794 and p=0.0153 for L-EV main effect; $F_{(1,13)}$=17.60 and p=0.0010 for CRST×L-EV; *Nt3*, $F_{(1,14)}$=43.45 and p<0.0001 for CRST main effect; $F_{(1,14)}$=3.274 and p=0.0919 for L-EV main effect; $F_{(1,14)}$=22.21 and p=0.0003 for CRST×L-EV; *Nt4/5*, $F_{(1,11)}$=0.0645 and p=0.8042 for CRST main effect; $F_{(1,11)}$=6.108 and p=0.0310 for L-EV main effect; $F_{(1,11)}$=15.76 and p=0.0022 for CRST×L-EV; *Trkb*, $F_{(1,14)}$=0.3743 and p=0.5505 for CRST main effect; $F_{(1,14)}$=7.106 and p=0.0185 for L-EV main effect; $F_{(1,14)}$=1.669 and p=0.2173 for CRST×L-EV). For the comparison of CON+veh, CRST+veh, CRST+L-EV, and CRST+IMI groups, one-way ANOVA followed by Newman-Keuls *post hoc* test was used (*Ngf*, $F_{(3,14)}$=1.495 and p=0.2591; t*Bdnf*, $F_{(3,14)}$=2.938 and p=0.0699; *Bdnf1*, $F_{(3,11)}$=14.81 and p=0.0004; *Bdnf4*, $F_{(3,13)}$=5.176 and p=0.0143; *Nt3*, $F_{(3,12)}$=9.203 and p=0.0020; *Nt4/5*, $F_{(3,11)}$=2.170 and p=0.1493; *Trkb*, $F_{(3,14)}$=3.280 and p=0.0527). (D) Western blot analysis of proBDNF and BDNF expression in the hippocampus of CON+veh, CON+L-EV, CRST+veh, and CRST+L-EV (n=8 animals and 3~5 WB repeats per group). Two-way ANOVA followed by Bonferroni *post hoc* test (proBDNF, $F_{(1,16)}$=22.62 and p=0.0002 for CRST main effect, $F_{(1,16)}$=4.830 and p=0.0430 for L-EV main effect, and $F_{(1,16)}$=3.681 and p=0.0731 for CRST×L-EV; BDNF, $F_{(1,8)}$=1.329 and p=0.2823 for CRST main effect, $F_{(1,8)}$=10.25 and p=0.0126 for L-EV main effect, and $F_{(1,8)}$=0.1521 and p=0.7067 for CRST×L-EV). (E~H) Behavior tests (#1) on post-stress days 14~16. Representative tracking of mice placed in the sociability test (E). The percent time (%) spent in the target or non-target fields (F), and immobility time in the TST (G) and FST (H) of CON+veh, CON+L-EV, CRST+veh, CRST+L-EV, and CRST+IMI (n=6~12 animals per group). Two-way ANOVA followed by Bonferroni *post hoc* test was used for the comparison of CON+veh, CON+L-EV, CRST+veh, CRST+L-EV (target field in the sociability test, $F_{(1,27)}$=2.243 and p=0.1458 for CRST main effect, $F_{(1,27)}$=0.6705 and p=0.4200 for L-EV main effect, and $F_{(1,27)}$=9.550 and p=0.0046 for CRST×L-EV; non-target field in the sociability test, $F_{(1,27)}$=2.420 and p=0.1315 for CRST main effect, $F_{(1,27)}$=0.5370 and p=0.4700 for L-EV main effect, and $F_{(1,27)}$=9.869 and p=0.0041 for CRST×L-EV; TST, $F_{(1,34)}$=7.324 and p=0.0106 for CRST main effect, $F_{(1,34)}$=0.6786 and p=0.4158 for L-EV main effect, and $F_{(1,34)}$=5.308 and p=0.0275 for CRST×L-EV; FST, $F_{(1,35)}$=29.00 and p<0.0001 for CRST main effect, $F_{(1,35)}$=5.846 and p=0.0210 for L-EV main effect, and $F_{(1,35)}$=18.19 and p=0.0001 for CRST×L-EV). One-way ANOVA followed by Neuman-Keuls *post hoc* test was for the comparison of CON+veh, CRST+veh, CRST+L-EV and CRST+IMI (target field in the sociability test, $F_{(3,27)}$=6.470 and p=0.0019; non-target field in the sociability test, $F_{(3,27)}$=6.435 and p=0.0020; TST, $F_{(3,33)}$=6.772 and p=0.001; FST, $F_{(3,34)}$=21.57 and p<0.0001). (I~L) Behavior tests (#2) on post-stress days 28~30. Representative tracking of mice placed in the sociability test (I). The percent time (%) spent in the target or non-target fields (J), and immobility time in the TST (K) and FST (L) of CON+veh, CON+L-EV, CRST+veh, CRST+L-EV, and CRST+IMI (n=6~12 animals per group). Two-way ANOVA followed by Bonferroni *post hoc* test was used for the comparison of CON+veh, CON+L-EV, CRST+veh, CRST+L-EV (target field in the sociability test, $F_{(1,28)}$=1.580 and p=0.2192 for CRST main effect, $F_{(1,28)}$=9.744 and p=0.0041 for L-EV main effect, and $F_{(1,28)}$=1.332 and p=0.2582 for CRST×L-EV; non-target field in the sociability test, $F_{(1,28)}$=1.557 and p=0.2224 for CRST main effect, $F_{(1,28)}$=9.706 and p=0.0042 for L-EV main effect, and $F_{(1,28)}$=1.304 and p=0.2632 for CRST×L-EV; TST, $F_{(1,36)}$=2.902 and p=0.0971 for CRST main effect, $F_{(1,36)}$=0.6042 and p=0.4421 for L-EV main effect, and $F_{(1,36)}$=6.737 and p=0.0136 for CRST×L-EV; FST, $F_{(1,35)}$=6.714 and p=0.0139 for CRST main effect, $F_{(1,35)}$=5.713 and p=0.0224 for L-EV main effect, and $F_{(1,35)}$=5.783 and p=0.0216 for CRST×L-EV). One-way ANOVA followed by Neuman-Keuls *post hoc* test was for the comparison of CON+veh, CRST+veh, CRST+L-EV and CRST+IMI (target field in the sociability test, $F_{(3,27)}$=4.567 and p=0.0103; non-target field in sociability test, $F_{(3,27)}$=4.535 and p=0.0106; TST, $F_{(3,31)}$=6.029 and p=0.0023; FST, $F_{(3,34)}$=5.762 and p=0.0027). Data are presented as mean±SEM. *p<0.05; **p<0.01.

maintained.

DISCUSSION

Lactobacillus-derived EVs increased BDNF expression in the hippocampus and rescued depressive-like behaviors

In the present study, we demonstrated that L-EV treatment in mice showing stress-induced depression increased BDNF expression in the hippocampus and produced antidepressant-like effects (Fig. 2 and 3). The results of antidepressant-like effects of L-EVs are consistent with the previous reports showing beneficial effects of *Lactobacillus spp.* on various mental disorders. For example, the supplement of *Lactobacillus rhamnosus* reduced stress-induced increase of corticosterone and anxiety- and depression-related behavior [8] and decreased stress-induced anxiety-like behavior and deficits in social interaction [51]. Treatment of *Lactobacil-lus helveticus* NS8 in hyperammonemia rats attenuated the level of inflammatory markers, restored cognitive function and improved anxiety-like behavior [52]. Furthermore, administration of *Lactobacillus helveticus* NS8 produced antidepressant effects in rats subjected to chronic restraint stress [10]. Oral ingestion of *Lactobacillus paracasei* in senescence accelerated mouse prone 8 (SAMP8) mice delayed age-related cognitive decline, possibly by preventing oxidation and inflammation [53]. *Lactobacillus plantarum* C29 treatment increased hippocampal BDNF and p-CREB expression and protected scopolamine-induced memory deficit in the Y-maze and Morris water maze tests [54]. Furthermore, supplementation of *Lactobacillus plantarum* prevented stress-induced depressive-like behaviors in mice [35], and attenuated anxiety-related behavior in zebrafish [34]. Thus, given that supplementation of *Lactobacillus spp.* produces beneficial effects on cognitive and emotional dysfunction in various experimental conditions, it is

important to note that *L*-EVs are sufficient to produce antidepressant effects. The antidepressant-like effects of *L*-EVs on behaviors were comparable to those of IMI (Fig. 3), whereas *L*-EV effects on the expression of neurotropic factors and TrkB were only partially overlapped with those of IMI, raising the possibility that *L*-EVs might exert antidepressant-like effects in a way slightly different from that of IMI, and therefore *L*-EVs could provide something that IMI does not afford.

The results of EVs-induced increase of BDNF and other genes in HT22 cells (Fig. 1) indicate that EVs can induce genomic responses by directly acting on cells. Considering that intraperitoneally (i.p.) injected *L*-EVs in mice induced genomic responses in the brain that were similar to those observed in HT22 cells (Fig. 1~3), it is possible that i.p.-injected EVs directly acted on the brain. It is well known that i.p.-injected drugs are normally absorbed into the mesenteric veins that are carried, via the hepatic portal vein, to the liver, and then they enter into the systemic circulatory system via the heart. It might be possible that injected *L*-EVs in mice immediately enter into the circulatory system. Consistent with this speculation, recent studies reported that fluorescent lipophilic dye-labeled EVs, when treated orally (p.o.), intravenously (i.v.), subcutaneously (s.c.) or intraperitoneally in all cases, are accumulated in many organs in the body, including the brain, liver, and kidney, although accumulation levels vary among different regions. For examples, DiD dye-labeled lung cancer cell (LL-2 cell)-derived EVs i.p. injected in C57BL/6 mice were detected in the brain 24 h after treatment [55]. *Staphylococcus aureus*-derived EVs [56] or *Helicobacter pylori*-derived EVs (100 μg/100 μl) [57] treated in C57BL/6 mice were also found in the brain 24 h after treatment.

Sirt1 played a role in L-EV-induced increased expression of BDNF and CREB in HT22 cells

L-EV treatment in HT22 cells increased BDNF expression, and this *L*-EV-induced BDNF expression was in part mediated by Sirt1, but not HDAC2 (Fig. 1). Thus, our results are consistent with the notion that *L*-EVs in HT22 cells induced BDNF expression through a transcriptional mechanism. *L*-EV treatment in CRST-treated mice also increased BDNF and NT4/5 expression in the hippocampus (Fig. 3C and 3D) and these *L*-EV effects were sustained for over 21 days after termination of *L*-EV treatment (Fig. 2C and 3C). These results suggest that *L*-EVs exert persistent changes in the expression of neurotropic factors.

BDNF expression is reduced in the hippocampus of postmortem samples from major depressive disorder patients and in mice with stress-induced depression [58, 59]. In contrast, depressive behavior is reversed by administration of recombinant BDNF in mice [60]. BDNF expression is regulated by epigenetic factors

such as Sirt1, HDACs and MeCP2 in stress-induced depression models [61, 62]. Sirt1 has a deacetylase activity [48]. Sirt1 indirectly regulates BDNF and CREB expression by decreasing miR-134 expression [48, 63]. Sirt1 can promote axon development and dendritic arborization [63-65]. Sirt1 expression is conversely regulated by miRNAs or other factors [48, 63, 66]. Sirt1 is reduced in the hippocampus of mice displaying depressive-like behaviors induced by chronic unpredictable mild stress (CUMS), whereas its activation reversed depression-like behaviors [64]. In rats exposed to CUMS, depression-like behaviors were reversed by resveratrol, which increased the expression of Sirt1, CREB, and BDNF in the hippocampus, while decreasing miR-134 [67]. As demonstrated in the present study, Sirt1 was also reduced in the hippocampus of mice exposed to chronic restraint stress, whereas *L*-EV treatment increased Sirt1 and BDNF (Fig. 2 and 3). *Sirt1* inhibition in HT22 cells reduced the expression of t*Bdnf*, *Bdnf1*, *Bdnf4*, and *Creb1*. *Sirt1* inhibition in *L*-EV-treated HT22 cells reduced the expression of *Bdnf4* and *Creb1*, compared to that of *L*-EV-treated HT22 cells, suggesting that *Sirt1* may have a role in *L*-EV-induced upregulation of *Bdnf4* and *Creb1* (Fig. 1D and 1E). However, *L*-EV-induced increase of t*Bdnf* and *Bdnf1* was not completely blocked by siRNA-Sirt1 (Fig. 1D and 1E). These results suggest that *L*-EVs might increase BDNF expression via Sirt1-dependent and Sirt1-independent mechanisms. MeCP2 is an epigenetic factor that can regulate BDNF expression [68, 69]. It may be worthwhile to examine whether MeCP2 is involved in *L*-EV effects on BDNF expression in neuronal cells.

Which factor contained in *L*-EVs increases the expression of *Sirt1* is not studied in the present study, but it is a challenging question that needs to be solved. EVs contain proteins, lipids, DNAs, RNAs, enzymes, bacterial metabolites, and toxins [70, 71]. The genus *Bacteroides* secreted EVs containing glycosidases and proteases that digested glycans which was used for growth [72]. *Bacteroides fragilis*-derived EVs contained PSA, which prevented colitis by enhancing immunomodulatory effects [73]. EVs derived from *Pseudomonas aeruginosa* contained bacteria DNAs, which were transferred to lung epithelial cells and were amplified by PCR in the nuclear fraction of cells [74]. Thus, bacterial EVs that contain proteins, lipids, DNA/RNAs, or bacterial metabolites might have a potential to exert physiological effects on host cells. Regarding the dramatic changes in gene expression alteration and behavioral rescues by EVs (Fig. 1~3), the detailed mechanisms how bacteria-derived EVs affect brain function remain to be explored further.

In conclusion, *L*-EV treatment increases the expression of BDNF in hippocampal neurons and produces antidepressant-like effects. Thus, our results support the notion that *L*-EVs and their direct action on neuronal cells mediate antidepressant effects of *Lacto-*

bacillus spp.

REFERENCES

1. Foster JA, McVey Neufeld KA (2013) Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci 36:305-312.

2. Rogers GB, Keating DJ, Young RL, Wong ML, Licinio J, Wesselingh S (2016) From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. Mol Psychiatry 21:738-748.

3. Aizawa E, Tsuji H, Asahara T, Takahashi T, Teraishi T, Yoshida S, Ota M, Koga N, Hattori K, Kunugi H (2016) Possible association of *Bifidobacterium* and *Lactobacillus* in the gut microbiota of patients with major depressive disorder. J Affect Disord 202:254-257.

4. Kelly JR, Borre Y, O'Brien C, Patterson E, El Aidy S, Deane J, Kennedy PJ, Beers S, Scott K, Moloney G, Hoban AE, Scott L, Fitzgerald P, Ross P, Stanton C, Clarke G, Cryan JF, Dinan TG (2016) Transferring the blues: depression-associated gut microbiota induces neurobehavioural changes in the rat. J Psychiatr Res 82:109-118.

5. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, Wang W, Tang W, Tan Z, Shi J, Li L, Ruan B (2015) Altered fecal microbiota composition in patients with major depressive disorder. Brain Behav Immun 48:186-194.

6. Akkasheh G, Kashani-Poor Z, Tajabadi-Ebrahimi M, Jafari P, Akbari H, Taghizadeh M, Memarzadeh MR, Asemi Z, Esmaillzadeh A (2016) Clinical and metabolic response to probiotic administration in patients with major depressive disorder: a randomized, double-blind, placebo-controlled trial. Nutrition 32:315-320.

7. Kato-Kataoka A, Nishida K, Takada M, Kawai M, Kikuchi-Hayakawa H, Suda K, Ishikawa H, Gondo Y, Shimizu K, Matsuki T, Kushiro A, Hoshi R, Watanabe O, Igarashi T, Miyazaki K, Kuwano Y, Rokutan K (2016) Fermented milk containing *Lactobacillus casei* strain Shirota prevents the onset of physical symptoms in medical students under academic examination stress. Appl Environ Microbiol 82:3649-3658.

8. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF (2011) Ingestion of *Lac-tobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 108:16050-16055.

9. McVey Neufeld KA, Kay S, Bienenstock J (2018) Mouse strain affects behavioral and neuroendocrine stress responses following administration of probiotic *Lactobacillus rhamnosus* JB-1 or traditional antidepressant fluoxetine. Front Neurosci 12:294.

10. Liang S, Wang T, Hu X, Luo J, Li W, Wu X, Duan Y, Jin F (2015) Administration of *Lactobacillus helveticus* NS8 improves behavioral, cognitive, and biochemical aberrations caused by chronic restraint stress. Neuroscience 310:561-577.

11. Marin IA, Goertz JE, Ren T, Rich SS, Onengut-Gumuscu S, Farber E, Wu M, Overall CC, Kipnis J, Gaultier A (2017) Microbiota alteration is associated with the development of stress-induced despair behavior. Sci Rep 7:43859.

12. Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13:701-712.

13. Carabotti M, Scirocco A, Maselli MA, Severi C (2015) The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Ann Gastroenterol 28:203-209.

14. Macfarlane S, Macfarlane GT (2003) Regulation of short-chain fatty acid production. Proc Nutr Soc 62:67-72.

15. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, Pettersson S (2012) Host-gut microbiota metabolic interactions. Science 336:1262-1267.

16. Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M (2011) Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun 25:397-407.

17. Belkaid Y, Hand TW (2014) Role of the microbiota in immunity and inflammation. Cell 157:121-141.

18. Schirmer M, Smeekens SP, Vlamakis H, Jaeger M, Oosting M, Franzosa EA, Ter Horst R, Jansen T, Jacobs L, Bonder MJ, Kurilshikov A, Fu J, Joosten LA, Zhernakova A, Huttenhower C, Wijmenga C, Netea MG, Xavier RJ (2016) Linking the human gut microbiome to inflammatory cytokine production capacity. Cell 167:1125-1136.e8.

19. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG (2008) The probiotic *Bifidobacteria infantis*: an assessment of potential antidepressant properties in the rat. J Psychiatr Res 43:164-174.

20. Lyte M (2011) Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design and use of probiotics. BioEssays 33:574-581.

https://doi.org/10.5607/en.2019.28.2.158

21. Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C (2012) γ-Aminobutyric acid production by culturable bacteria from the human intestine. J Appl Microbiol 113:411-417.

22. Duerkop BA, Vaishnava S, Hooper LV (2009) Immune responses to the microbiota at the intestinal mucosal surface. Immunity 31:368-376.

23. de Lartigue G, de La Serre CB, Raybould HE (2011) Vagal afferent neurons in high fat diet-induced obesity; intestinal microflora, gut inflammation and cholecystokinin. Physiol Behav 105:100-105.

24. Breit S, Kupferberg A, Rogler G, Hasler G (2018) Vagus nerve as modulator of the brain-gut axis in psychiatric and inflammatory disorders. Front Psychiatry 9:44.

25. Ahmadi Badi S, Moshiri A, Fateh A, Rahimi Jamnani F, Sarshar M, Vaziri F, Siadat SD (2017) Microbiota-derived extracellular vesicles as new systemic regulators. Front Microbiol 8:1610.

26. van Niel G, D'Angelo G, Raposo G (2018) Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol 19:213-228.

27. Chelakkot C, Choi Y, Kim DK, Park HT, Ghim J, Kwon Y, Jeon J, Kim MS, Jee YK, Gho YS, Park HS, Kim YK, Ryu SH (2018) *Akkermansia muciniphila*-derived extracellular vesicles influence gut permeability through the regulation of tight junctions. Exp Mol Med 50:e450.

28. Kang CS, Ban M, Choi EJ, Moon HG, Jeon JS, Kim DK, Park SK, Jeon SG, Roh TY, Myung SJ, Gho YS, Kim JG, Kim YK (2013) Extracellular vesicles derived from gut microbiota, especially *Akkermansia muciniphila*, protect the progression of dextran sulfate sodium-induced colitis. PLoS One 8:e76520.

29. Shen Y, Giardino Torchia ML, Lawson GW, Karp CL, Ashwell JD, Mazmanian SK (2012) Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. Cell Host Microbe 12:509-520.

30. Goffin P, van de Bunt B, Giovane M, Leveau JH, Höppener-Ogawa S, Teusink B, Hugenholtz J (2010) Understanding the physiology of *Lactobacillus plantarum* at zero growth. Mol Syst Biol 6:413.

31. Anjum N, Maqsood S, Masud T, Ahmad A, Sohail A, Momin A (2014) *Lactobacillus acidophilus*: characterization of the species and application in food production. Crit Rev Food Sci Nutr 54:1241-1251.

32. Paolillo R, Romano Carratelli C, Sorrentino S, Mazzola N, Rizzo A (2009) Immunomodulatory effects of *Lactobacillus plantarum* on human colon cancer cells. Int Immunopharmacol 9:1265-1271.

33. Tian F, Yu L, Zhai Q, Xiao Y, Shi Y, Jiang J, Liu X, Zhao J, Zhang H, Chen W (2017) The therapeutic protection of a living and dead *Lactobacillus* strain against aluminum-induced brain and liver injuries in C57BL/6 mice. PLoS One 12:e0175398.

34. Davis DJ, Doerr HM, Grzelak AK, Busi SB, Jasarevic E, Ericsson AC, Bryda EC (2016) *Lactobacillus plantarum* attenuates anxiety-related behavior and protects against stress-induced dysbiosis in adult zebrafish. Sci Rep 6:33726.

35. Dhaliwal J, Singh DP, Singh S, Pinnaka AK, Boparai RK, Bishnoi M, Kondepudi KK, Chopra K (2018) *Lactobacillus plantarum* MTCC 9510 supplementation protects from chronic unpredictable and sleep deprivation-induced behaviour, biochemical and selected gut microbial aberrations in mice. J Appl Microbiol 125:257-269.

36. Kim MH, Choi SJ, Choi HI, Choi JP, Park HK, Kim EK, Kim MJ, Moon BS, Min TK, Rho M, Cho YJ, Yang S, Kim YK, Kim YY, Pyun BY (2018) *Lactobacillus plantarum*-derived extracellular vesicles protect atopic dermatitis induced by *Staphylococcus aureus*-derived extracellular vesicles. Allergy Asthma Immunol Res 10:516-532.

37. Seo JS, Park JY, Choi J, Kim TK, Shin JH, Lee JK, Han PL (2012) NADPH oxidase mediates depressive behavior induced by chronic stress in mice. J Neurosci 32:9690-9699.

38. Kim TK, Han PL (2016) Functional connectivity of basolateral amygdala neurons carrying orexin receptors and melanin-concentrating hormone receptors in regulating sociability and mood-related behaviors. Exp Neurobiol 25:307-317.

39. Han HE, Kim TK, Son HJ, Park WJ, Han PL (2013) Activation of autophagy pathway suppresses the expression of iNOS, IL6 and eell death of LPS-stimulated microglia cells. Biomol Ther (Seoul) 21:21-28.

40. Choi J, Kim JE, Kim TK, Park JY, Lee JE, Kim H, Lee EH, Han PL (2015) TRH and TRH receptor system in the basolateral amygdala mediate stress-induced depression-like behaviors. Neuropharmacology 97:346-356.

41. Kim TK, Kim JE, Choi J, Park JY, Lee JE, Lee EH, Lee Y, Kim BY, Oh YJ, Han PL (2017) Local interleukin-18 system in the basolateral amygdala regulates susceptibility to chronic stress. Mol Neurobiol 54:5347-5358.

42. Park JY, Kim TK, Choi J, Lee JE, Kim H, Lee EH, Han PL (2014) Implementation of a two-dimensional behavior matrix to distinguish individuals with differential depression states in a rodent model of depression. Exp Neurobiol 23:215-223.

43. Maqsood R, Stone TW (2016) The gut-brain axis, BDNF, NMDA and CNS disorders. Neurochem Res 41:2819-2835.

44. Distrutti E, O'Reilly JA, McDonald C, Cipriani S, Renga B, Lynch MA, Fiorucci S (2014) Modulation of intestinal microbiota by the probiotic VSL#3 resets brain gene expression

and ameliorates the age-related deficit in LTP. PLoS One 9:e106503.

45. Ait-Belgnaoui A, Colom A, Braniste V, Ramalho L, Marrot A, Cartier C, Houdeau E, Theodorou V, Tompkins T (2014) Probiotic gut effect prevents the chronic psychological stress-induced brain activity abnormality in mice. Neurogastroenterol Motil 26:510-520.

46. Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME (1997) CREB: a major mediator of neuronal neurotrophin responses. Neuron 19:1031-1047.

47. Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, Gao J, Nieland TJ, Zhou Y, Wang X, Mazitschek R, Bradner JE, DePinho RA, Jaenisch R, Tsai LH (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459:55-60.

48. Herskovits AZ, Guarente L (2014) SIRT1 in neurodevelopment and brain senescence. Neuron 81:471-483.

49. Jeong H, Cohen DE, Cui L, Supinski A, Savas JN, Mazzulli JR, Yates JR 3rd, Bordone L, Guarente L, Krainc D (2011) Sirt1 mediates neuroprotection from mutant huntingtin by activation of the TORC1 and CREB transcriptional pathway. Nat Med 18:159-165.

50. Han A, Sung YB, Chung SY, Kwon MS (2014) Possible additional antidepressant-like mechanism of sodium butyrate: targeting the hippocampus. Neuropharmacology 81:292-302.

51. Bharwani A, Mian MF, Surette MG, Bienenstock J, Forsythe P (2017) Oral treatment with *Lactobacillus rhamnosus* attenuates behavioural deficits and immune changes in chronic social stress. BMC Med 15:7.

52. Luo J, Wang T, Liang S, Hu X, Li W, Jin F (2014) Ingestion of *Lactobacillus* strain reduces anxiety and improves cognitive function in the hyperammonemia rat. Sci China Life Sci 57:327-335.

53. Huang SY, Chen LH, Wang MF, Hsu CC, Chan CH, Li JX, Huang HY (2018) *Lactobacillus paracasei* PS23 delays progression of age-related cognitive decline in senescence accelerated mouse prone 8 (SAMP8) mice. Nutrients 10:E894.

54. Jung IH, Jung MA, Kim EJ, Han MJ, Kim DH (2012) *Lactobacillus pentosus* var. plantarum C29 protects scopolamine-induced memory deficit in mice. J Appl Microbiol 113:1498-1506.

55. Garofalo M, Villa A, Rizzi N, Kuryk L, Mazzaferro V, Ciana P (2018) Systemic administration and targeted delivery of immunogenic oncolytic adenovirus encapsulated in extracellular vesicles for cancer therapies. Viruses 10:558.

56. Choi SJ, Kim MH, Jeon J, Kim OY, Choi Y, Seo J, Hong SW, Lee WH, Jeon SG, Gho YS, Jee YK, Kim YK (2015) Active im-

munization with extracellular vesicles derived from *Staphylococcus aureus* effectively protects against staphylococcal lung infections, mainly via Th1 cell-mediated immunity. PLoS One 10:e0136021.

57. Choi HI, Choi JP, Seo J, Kim BJ, Rho M, Han JK, Kim JG (2017) *Helicobacter pylori*-derived extracellular vesicles increased in the gastric juices of gastric adenocarcinoma patients and induced inflammation mainly via specific targeting of gastric epithelial cells. Exp Mol Med 49:e330.

58. Yu H, Chen ZY (2011) The role of BDNF in depression on the basis of its location in the neural circuitry. Acta Pharmacol Sin 32:3-11.

59. Duclot F, Kabbaj M (2015) Epigenetic mechanisms underlying the role of brain-derived neurotrophic factor in depression and response to antidepressants. J Exp Biol 218:21-31.

60. Shirayama Y, Chen AC, Nakagawa S, Russell DS, Duman RS (2002) Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. J Neurosci 22:3251-3261.

61. Boulle F, van den Hove DL, Jakob SB, Rutten BP, Hamon M, van Os J, Lesch KP, Lanfumey L, Steinbusch HW, Kenis G (2012) Epigenetic regulation of the BDNF gene: implications for psychiatric disorders. Mol Psychiatry 17:584-596.

62. Fuchikami M, Yamamoto S, Morinobu S, Takei S, Yamawaki S (2010) Epigenetic regulation of BDNF gene in response to stress. Psychiatry Investig 7:251-256.

63. Gao J, Wang WY, Mao YW, Gräff J, Guan JS, Pan L, Mak G, Kim D, Su SC, Tsai LH (2010) A novel pathway regulates memory and plasticity via SIRT1 and miR-134. Nature 466:1105-1109.

64. Abe-Higuchi N, Uchida S, Yamagata H, Higuchi F, Hobara T, Hara K, Kobayashi A, Watanabe Y (2016) Hippocampal sirtuin 1 signaling mediates depression-like behavior. Biol Psychiatry 80:815-826.

65. Li XH, Chen C, Tu Y, Sun HT, Zhao ML, Cheng SX, Qu Y, Zhang S (2013) Sirt1 promotes axonogenesis by deacetylation of Akt and inactivation of GSK3. Mol Neurobiol 48:490-499.

66. Ng F, Wijaya L, Tang BL (2015) SIRT1 in the brain-connections with aging-associated disorders and lifespan. Front Cell Neurosci 9:64.

67. Shen J, Xu L, Qu C, Sun H, Zhang J (2018) Resveratrol prevents cognitive deficits induced by chronic unpredictable mild stress: Sirt1/miR-134 signalling pathway regulates CREB/BDNF expression in hippocampus *in vivo* and *in vitro*. Behav Brain Res 349:1-7.

68. Zhou Z, Hong EJ, Cohen S, Zhao WN, Ho HY, Schmidt L, Chen WG, Lin Y, Savner E, Griffith EC, Hu L, Steen JA, Weitz

CJ, Greenberg ME (2006) Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation. Neuron 52:255-269.

69. Klein ME, Lioy DT, Ma L, Impey S, Mandel G, Goodman RH (2007) Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA. Nat Neurosci 10:1513-1514.

70. Schwechheimer C, Kuehn MJ (2015) Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol 13:605-619.

71. Fateh A, Vaziri F, Rahimi Janani F, Ahmadi Badi S, Ghazanfari M, Davari M, Arsang A, Siadat SD (2016) New insight into the application of outer membrane vesicles of Gram-negative bacteria. Vaccine Res 3:1-4.

72. Elhenawy W, Debelyy MO, Feldman MF (2014) Preferential packing of acidic glycosidases and proteases into *Bacteroides* outer membrane vesicles. MBio 5:e00909-e00914.

73. Mazmanian SK, Round JL, Kasper DL (2008) A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 453:620-625.

74. Bitto NJ, Chapman R, Pidot S, Costin A, Lo C, Choi J, D'Cruze T, Reynolds EC, Dashper SG, Turnbull L, Whitchurch CB, Stinear TP, Stacey KJ, Ferrero RL (2017) Bacterial membrane vesicles transport their DNA cargo into host cells. Sci Rep 7:7072.