

Humulus japonicus rescues autistic-like behaviours in the BTBR T⁺ Itpr3^{tf}/J mouse model of autism

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Abstract. *Humulus japonicus* (HJ) is a traditional herbal medicine that exhibits anti-inflammatory, antimicrobial and anti-tumor effects that is used for the treatment of hypertension, pulmonary disease and leprosy. Recently, it has also been reported that HJ demonstrates neuroprotective properties in animal models of neurodegenerative diseases. The current study hypothesised that the administration of HJ would exhibit therapeutic effects in autism spectrum disorder (ASD), a neurodevelopmental disorder with lifelong consequences. The BTBR T⁺ Itpr3^{tf}/J mouse model of ASD was used to investigate the anti-autistic like behavioural effects of HJ. Chronic oral administration of the ethanolic extract of HJ significantly increased social interaction, attenuated repetitive grooming behaviour and improved novel-object recognition in BTBR mice. Anti-inflammatory effects of HJ in the brain were analysed using immunohistochemistry and reverse-transcription quantitative PCR analysis. Microglia activation was markedly decreased in the striatum and hippocampus, and pro-inflammatory cytokines, including C-C Motif Chemokine Ligand 2, interleukin (IL)-1 β and IL-6, were significantly reduced in the hippocampus following HJ treatment. Moreover, HJ treatment normalised the phosphorylation levels of: N-methyl-D-aspartate receptor subtype 2B and calcium/calmodulin-dependent protein kinase type II subunit

α in the hippocampus of BTBR mice. The results of the present study demonstrated that the administration of HJ may have beneficial potential for ameliorating behavioural deficits and neuroinflammation in ASD.

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that includes a social interaction deficit and restrictive and repetitive behavioural patterns (DSM-5). Besides the key symptoms, cognitive deficits are frequently present in patients with ASD (1). ASD affects approximately 1 in 54 people in the United States of America (CDC, 2020). Despite the fact that a significant proportion of children are diagnosed with ASD, no effective treatment exists for core ASD symptoms.

Mouse models with face validity to the core symptoms, such as a social interaction deficit and restrictive and repetitive behavioural patterns, have offered experimental approaches to evaluate potential treatments for ASD. Inbred BTBR T⁺ Itpr3^{tf}/J (BTBR) mice exhibited reduced sociability and increased self-grooming behaviour, which mimics the core symptoms of social interaction deficits and repetitive behaviours seen in ASD patients (2,3). In addition, BTBR mice also exhibited object-based attention deficits (4), and complete absence of corpus callosum, which is comparable to ASD patients with reduced volume of the corpus callosum (5,6). Alterations in glia, neurons, and synapses and reduction in neurogenesis in the brain of BTBR mice have also been reported (7).

Microglia are well-known immune cells of the central nervous system. Maternal immune activation and increased pro-inflammatory cytokines lead to a higher risk of ASD (8). A growing body of evidence indicates that microglial dysfunction is a potential target for ASD (9). In human post-mortem studies, increased gliosis and glial cell proliferation have been reported in patients with ASD (10). Immunomodulatory treatments such as vitamin D, suramin, minocycline, and gut microbiota effectively improved autistic-like behaviours and decreased pro-inflammatory cytokines (11). In addition,

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neuroinflammation has been linked to synaptic loss, and microglia play critical roles in activity-dependent synapse remodelling (12).

Humulus japonicus (HJ) is a perennial herb distributed in Asian countries. HJ has traditionally been used in patients with hypertension, pulmonary disease, and skin disease in Korea. Recently, the protective effects of HJ on neurodegenerative diseases involving Alzheimer's disease (AD) and Parkinson's disease (PD) have been reported in *in vivo* animal studies (13,14). HJ has demonstrated anti-inflammatory effects on paw oedema, AD, and PD animal models (13-15). HJ has also been reported to significantly improve the cognitive function of APP/PS1 transgenic mice for AD (13). However, the effects of HJ on neurodevelopmental disorders and ASD have not yet been elucidated. In this study, we employed BTBR mice to investigate the hypothesis that autism-like behaviours could be ameliorated by HJ treatment. We also examined the effects of HJ on microglia activation, inflammatory cytokines, and the excitatory signalling pathway in the brain of BTBR mice.

Materials and methods

Animals. Male C57LB/6J (B6 mice) and BTBR T⁺ Itpr3^{tf}/J inbred strains (BTBR mice) were obtained from the Korea Research Institute of Bioscience and Biotechnology and the Jackson Laboratory (Bar Harbor, ME, USA). BTBR mice were maintained with BTBR x BTBR mice and their progenies were used in the study. Mice were housed in plastic cages (25x20x12.5 cm³) in a humidity-(50%-60%) and temperature-controlled (21-22°C) environment under specific pathogen-free conditions on a 12-h light/dark cycle (lights on at 07:00) with free access to autoclaved food and water. The microbiological status of the mice was monitored once every three months and serological investigations for viral infection were performed every month. Animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Use and Care Committee of the Korea Research Institute of Bioscience and Biotechnology. Mice were randomised into vehicle and HJ-treated groups at the age of 3 weeks to the following: B6 group treated with vehicle alone (n=12), B6 group treated with 400 mg/kg HJ (n=6), BTBR mice treated with vehicle alone (n=13), BTBR mice treated with 200 mg/kg HJ (n=13), and BTBR mice treated with 400 mg/kg HJ (n=13). HJ and 0.5% carboxymethyl cellulose (CMC, vehicle) were administered by oral gavage for 6 weeks daily.

Preparation of HJ. HJ was purchased from Gangwon Herbs, Gangwon Province, Republic of Korea, in July 2014. The voucher specimen was identified by Professor WK. Oh, and a voucher specimen (SNU-2014-0004) was deposited at the College of Pharmacy, Seoul National University, Korea. Then, the HJ extract was prepared and supplied by the Korea Bioactive Natural Material Bank (Seoul, Korea). The dried aerial parts of HJ were soaked in 20% ethanol in an extraction container for 2 days at room temperature. The ethanol-soluble extracts of HJ were filtered through cheesecloth, concentrated exhaustively, and dried to produce an ethanolic extract under reduced

pressure. The 20% ethanol extract of HJ was used in this study. The HJ extract was suspended in 0.5% CMC at a concentration of 50 mg/ml as a stock solution, and the working solution of HJ was adjusted to the intended concentrations for use in *in vivo* experiments.

Behavioural tests. Behavioural tests were performed between 4 and 6 weeks of HJ and vehicle treatment. Vehicle or drugs were administered 30 min before all behavioural tests. The mice were acclimatised to the test room for 30 min prior to each test. The following tests were sequentially performed: Open field test → self-grooming test → novel object recognition test → social interaction test). No more than one test was performed on any given day (Fig. 1).

Open field test. General exploration of mice was performed using an open field test, which has been described in previous studies (16). Each mouse was gently introduced into a white plexiglass chamber (45x45x40 cm), and the horizontal locomotor activity was monitored simultaneously for 5 min using SMART video tracking software (Panlab, Barcelona, Spain).

Self-grooming test. Spontaneous self-grooming behaviour has been described in previous studies (17,18). Each mouse was gently introduced into a transparent acrylic cylinder (diameter, 20 cm). Repetitive self-grooming behaviours were recorded for 30 min using a video camera (Samsung, Korea). Grooming behaviour included head washing, body grooming, genital/tail grooming, and paw and leg licking. The cylinders were cleaned with 70% ethanol between each subject's test session.

Novel object recognition test. The novel-object recognition test has been described in previous studies (16). Mice were individually habituated in the testing chamber (40x20x20 cm) for 5 min. After placing 2 identical objects (familiar objects, cylindrical wooden blocks, 10 cm high x2 cm diameter), mice were allowed to move freely for 10 min. The mice were then returned to their cages. Twenty-four hours later, mice were placed back into the testing chamber in the presence of one of the familiar objects and one novel object (rectangular wooden block, 10x2.5x2 cm) for 10 min. The sessions were video-recorded, and the time spent exploring the objects was scored. The objects and chambers were cleaned with 70% ethanol between each session. Sniffing and touching the object with the nose and/or forepaws were defined as exploration. Sitting on the object was not considered an exploratory behaviour.

Social interaction test. The social interaction test has been described in previous studies (19). The social interaction chamber consisted of a white acryl wall box (40x20x20 cm). Individual mice were placed in the chamber for 3 min for habituation. Then, an age-matched novel C57BL/6J male mouse was introduced into the test chamber and allowed to explore freely for 3 min. The behaviours of the mice were video-recorded, and social interaction, such as body sniffing, anogenital sniffing, and direct contact were analysed for 3 min.

Reverse-transcription quantitative PCR. Thirty minutes after the 6-week HJ (400 mg/kg) treatment schedule, mice were euthanized by quick cervical dislocation approved by the

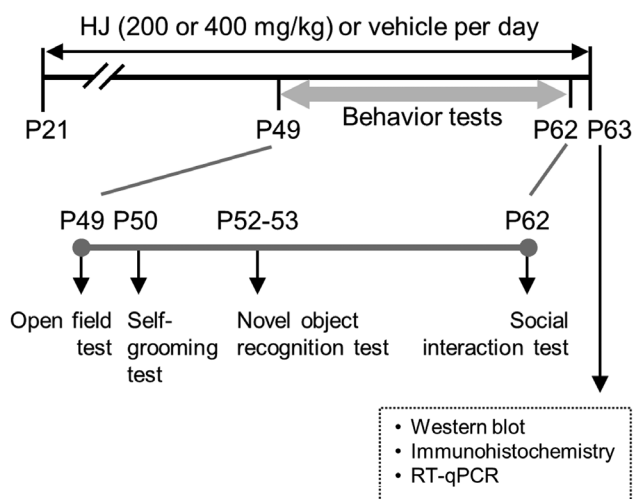


Figure 1. Experimental procedure. Experimental design for the administration of HJ, behavioural testing (open field test, self-grooming test, novel object recognition test and social interaction test) and sampling. HJ, *Humulus japonicus*.

IACUC and their brains are removed. Total RNA was extracted from the hippocampus using TRI reagent (Sigma-Aldrich; Merck KGaA). cDNA synthesis was performed using the Reverse Transcription System (Promega) according to the instructions of the supplier. RT-PCR analysis used in Fig. 5 was carried out using the following primer sets: IL-1b (5'-CTACAG GCTCCGAGATGAACAAC-3' and 5'-TCCATTGAGGTG GAGAGCTTTC-3'), IL6 (5'-TTCCATCCAGTTGCCTTCTTG-3' and 5'-GGGAGTGGTATCCTCTGTGAAGTC-3'), CCL2 (5'-TTAAAACCTGGATCGGAACCAA-3' and 5'-GCA TTAGCTTCAGATTTACGGGT-3'), CXCL10 (5'-CCAAGT GCTGCCGTCATTTTC-3' and 5'-GGCTCGCAGGGATGA TTTCAA-3'), and 18s ribosomal RNA (18s, 5'-GACACGGAC AGGATTGACAGATTGATAG-3' and 5'-GTTAGCATGCCA GAGTCTCGTTCGTT-3'). Comparative qPCR was performed using an SYBR Green Master Mix (Applied Biosystems). The expression level of target genes was normalized to the expression 18s (20) and calculated based on the comparative cycle threshold C_t method ($2^{-\Delta\Delta C_t}$) (21).

Western blotting. Western blotting was conducted as previously described (16). Thirty minutes after the 6-week HJ (400 mg/kg) treatment schedule, mice were euthanized by quick cervical dislocation and their brains were removed. Left hippocampus was homogenised in homogenisation buffer (1X RIPA buffer, Cat# 02-188; Millipore) containing a cocktail of protease inhibitors (Roche). The homogenates were centrifuged at 600 x g at 4°C for 10 min and the supernatants were centrifuged at 17,000 x g at 4°C for 10 min to obtain the supernatant containing the cytosolic fraction. Protein samples were resolved by SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane (Bio-Rad). Blots were incubated overnight at 4°C with the following primary antibodies: N-methyl-D-aspartate receptor subtype 2B (NR2B, Cat# 06-600; Millipore), pNR2B (Tyr1472, Cat# 4212; Cell Signaling Technology), calcium/calmodulin-dependent protein kinase type II subunit alpha (CaMKII α , Cat# sc-13141; Santa Cruz Biotechnology), pCaMKII α (Thr286, Cat# sc-12886; Santa Cruz Biotechnology), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor

subunit GluR1 (GluR1, Cat# 31232; Millipore), pGluR1 (Ser831, Cat# 04-823; Millipore), pGluR1 (Ser 845, Cat# 04-1073; Millipore), gamma-aminobutyric acid (GABA) receptor subunits: $\alpha 1$ (Cat# ab33299, Abcam), $\beta 2/3$ (Cat# 05-474, Millipore), and $\gamma 2$ (Cat# ab240445; Abcam), and actin (Cat# MAB1501; Millipore).

Immunohistochemistry. Immunohistochemistry was performed as previously described (22). Mice were transcardially perfused with saline followed by 4% paraformaldehyde in phosphate-buffered saline. The perfused brains were dissected, post-fixed overnight, and then cut into 40 μ m coronal sections on a vibratome (Leica). Free-floating sections were blocked with serum for 1 h and incubated overnight at 4°C with the primary rabbit polyclonal antibody for Iba-1 (ionised calcium-binding adaptor molecule 1, Cat# 019-19741; Wako). Immunohistochemistry was then performed using biotinylated secondary anti-rabbit IgG (Vector Laboratory), avidin-biotinylated peroxidase complex (Vector Laboratory), and 3,3'-diaminobenzidine (Sigma-Aldrich; Merck KGaA). The occupied areas of Iba-1 positive cells were measured in the cerebral cortex, hippocampus, and dorsal striatum. Images were taken at one or two optical planes per section at x200 magnification (Olympus Corporation; 10x ocular and 20x objective). Qualitative evaluations of immunoreactivity were performed in a blinded manner.

Statistical analysis. GraphPad Prism software (GraphPad Software, Inc.) was used to perform all statistical analyses. Two-sample comparisons were conducted with Student's t-tests, while multiple comparisons were performed with a one-way ANOVA followed by Tukey-Kramer post hoc tests. All data are presented as the mean \pm standard error of the mean (SEM). Differences with a P-value <0.05 were considered statistically significant.

Results

Self-grooming behaviour is decreased by HJ treatment in BTBR mice. To investigate the effect of HJ on locomotor activity and repetitive behaviours, we performed the open field test and cylinder test in the vehicle-treated or HJ-treated B6 and BTBR mice. One-way ANOVA analysis of distance travelled revealed that the BTBR mice moved more than the B6 control mice in the open field test (Fig. 2A, $P < 0.05$). However, HJ treatment did not alter locomotor activity in either B6 or BTBR mice. In the cylinder test, total self-grooming behaviours were significantly increased in vehicle-treated BTBR mice compared to vehicle-treated B6 mice (Fig. 2B, $P < 0.01$). The increased self-grooming in the BTBR mice was significantly decreased in the 400 mg/kg HJ-treated group compared to the vehicle-treated group (Fig. 2B, $P < 0.01$), but not in B6 mice. The results suggested that the repetitive behaviour was ameliorated by HJ treatment in BTBR mice.

Social interaction is increased by HJ treatment in BTBR mice. To determine the effect of HJ on social deficits, we performed a social interaction test in the vehicle-treated or HJ-treated B6 and BTBR mice. The total duration and number of sniffings in the sociability session were significantly decreased in the

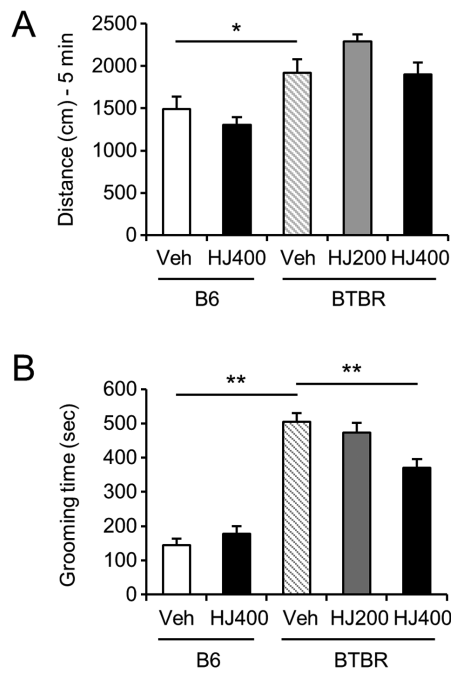


Figure 2. Effects of HJ treatment on locomotor activity and self-grooming behaviour in BTBR mice. (A) Total exploratory locomotion in the open field test showed a 5-min assay of distance moved. (B) Total self-grooming behaviour in the cylinder test showed a 30-min assay of head washing, body grooming, genital/tail grooming, paw and leg licking. * $P < 0.05$ and ** $P < 0.01$ vs. indicated group. Data are presented as mean \pm SEM. Veh, vehicle-treated group; HJ200, 200 mg/kg HJ-treated group; HJ400, 400 mg/kg HJ-treated group. HJ, *Humulus japonicus*.

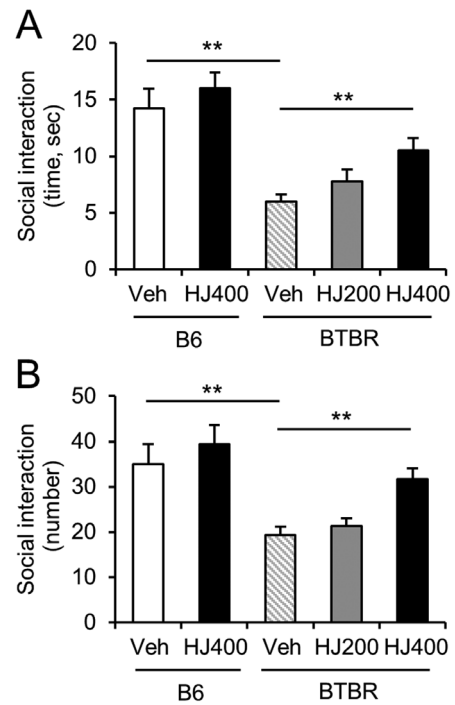


Figure 3. Effects of HJ treatment on social interaction behaviour in BTBR mice. Social interactions (A) time and (B) number, such as body sniffing, anogenital sniffing, and direct contact was measured in the vehicle-treated group and HJ-treated group. ** $P < 0.01$, vs. indicated group. Data are presented as mean \pm SEM. HJ, *Humulus japonicus*; Veh, vehicle-treated group; HJ200, 200 mg/kg HJ-treated group; HJ400, 400 mg/kg HJ-treated group.

vehicle-treated BTBR mice compared to vehicle-treated B6 mice (Fig. 3A and B, $P < 0.01$). We found significant effects of 400 mg/kg HJ treatment in BTBR mice, but not B6 mice (Fig. 3A and B, $P < 0.01$). BTBR mice treated with 400 mg/kg HJ exhibited significantly more time and number of sniffings in the social interaction test than the vehicle-treated BTBR mice (Fig. 3A; $F_{(4,47)} = 0.9223$, $P = 0.0004$; Fig. 3B, $F_{(4,47)} = 1.444$, $P < 0.0001$). This finding indicates that the administration of HJ markedly improved the sociability of BTBR mice.

Cognitive deficit is improved by HJ treatment in BTBR mice. To investigate whether impaired cognitive function in BTBR mice is improved by HJ treatment, we performed a novel object recognition test. We measured the total time spent sniffing and the total number of contacts with the novel object in each group (Fig. 3A and B). B6 mice spent significantly more time and had a significantly higher number of contacts with novel objects compared to the familiar object. B6 mice treated with HJ showed no significant difference in the total time spent sniffing and in contact with the novel object (Fig. 4A and B). Vehicle-treated BTBR mice did not show a preference for the novel object. However, 200 mg/kg or 400 mg/kg HJ treatment rescued the preference for the novel object (Fig. 4A and B, $P < 0.01$). These results suggest that HJ can improve cognitive function in BTBR mice.

Microglial activation is reduced in the cortex, hippocampus, and dorsal striatum. Since the anti-inflammatory effects of HJ on neurodegenerative disorders such as AD and PD have been reported (13,14), we also investigated the effects of HJ on

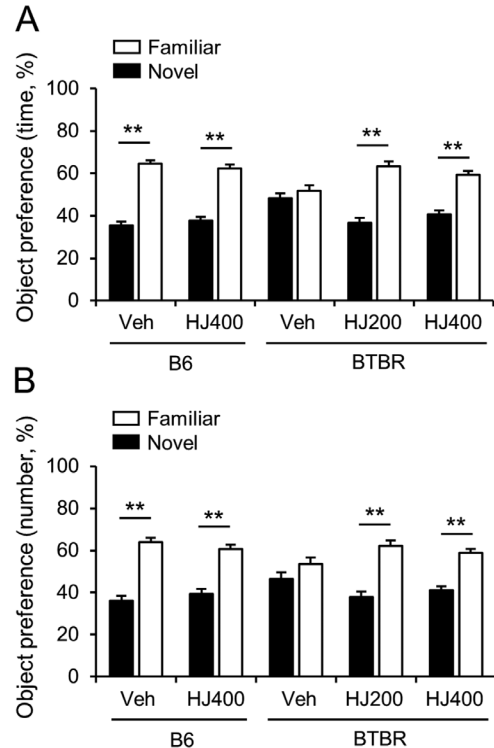


Figure 4. Effects of HJ treatment on novel object recognition in BTBR mice. The exploration preference of a novel object during the recognition session of the object recognition test was calculated. (A) Percentage of time of sniffing and touching the object with the nose and/or forepaws; (B) Number (%) of sniffing and touching the object with the nose and/or forepaws. ** $P < 0.01$ vs. indicated group. Data are presented as mean \pm SEM. HJ, *Humulus japonicus*; Veh, vehicle-treated group; HJ200, 200 mg/kg HJ-treated group; HJ400, 400 mg/kg HJ-treated group.

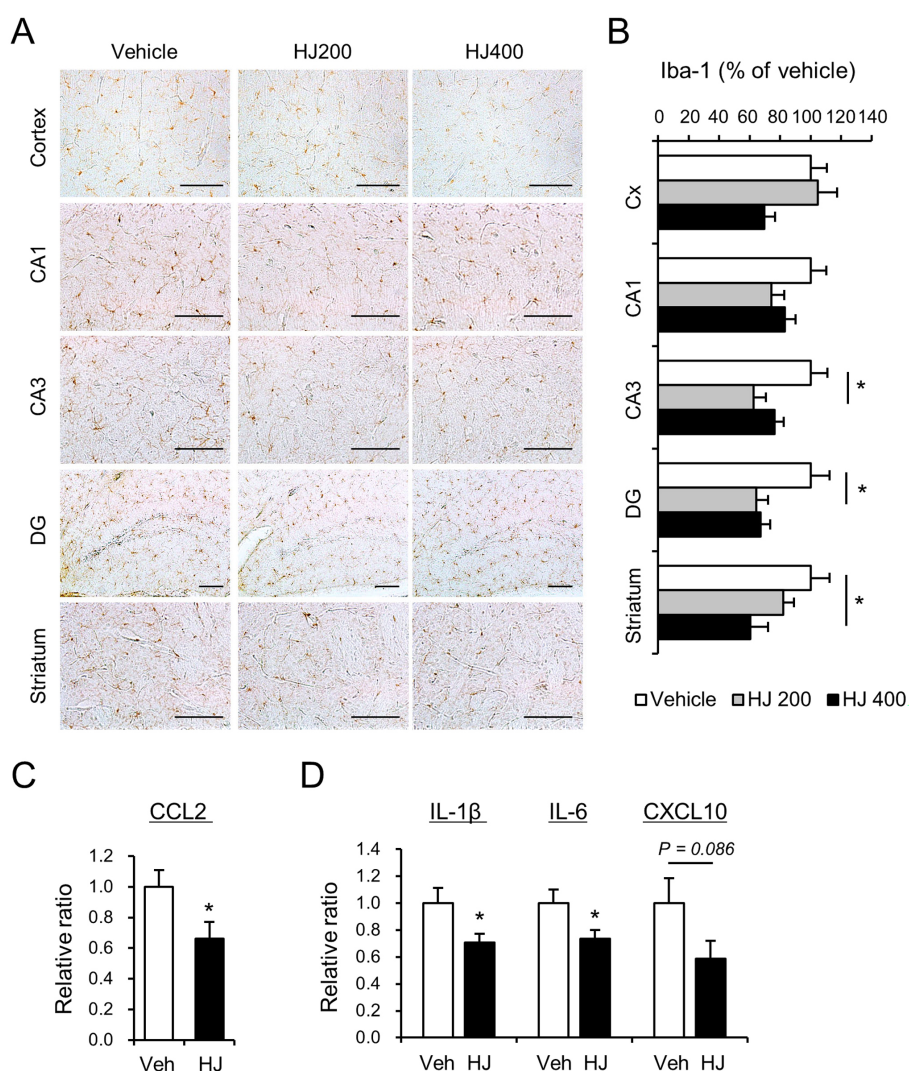


Figure 5. Effects of HJ treatment on the activation of microglia and the expression level of pro-inflammatory cytokines in BTBR mice. (A) Micrograph representation of the cerebral cortex, hippocampus (CA1, CA3 and DG), and striatum stained for Iba-1 in vehicle-treated (vehicle), 200 mg/kg HJ-treated (HJ200) and 400 mg/kg HJ-treated (HJ400) BTBR mice. Quantification of positive area stained is represented for the cerebral cortex (Cx), hippocampus (CA1, CA3 and DG), and (B) striatum for Iba-1. Scale bar represents 200 μ m in all images. (C and D) mRNA expression levels of cytokines following HJ treatment in the hippocampus of BTBR mice. Relative mRNA levels of (C) CCL2, (D) IL-1 β , IL-6 and CXCL10 were measured using reverse-transcription quantitative PCR. *P<0.05 vs. indicated group. Data are presented as mean \pm SEM. HJ, *Humulus japonicus*; CCL2, chemokine CC motif ligand 2; IL, interleukin.

microglial activation in the brain of BTBR mice. The percentage area occupied by Iba-1 immunoreactive cells were analysed in cerebral cortex, hippocampus, and striatum. HJ treatment (200 mg/kg) significantly decreased the percentage of occupied Iba-1 immunoreactive cells in the CA1, CA3, and DG of the hippocampus (Fig. 5A and B). HJ treatment (400 mg/kg) markedly decreased the percentage of occupied Iba-1 immunoreactive cells in the cerebral cortex, DG, and striatum (Fig. 5A and B). These results suggest that HJ can influence microglial activation in the cerebral cortex, hippocampus, and striatum.

The levels of cytokines and chemokines in patients with ASD have been shown to be altered compared to those in typically developing children (23). In particular, chemokine CC motif ligand 2 (CCL2) is significantly increased in patients with ASD (23). To determine the effect of HJ on the expression of CCL2 in the hippocampus, we performed quantitative RT-PCR analysis. The administration of HJ significantly decreased the mRNA expression of CCL2 in the hippocampus of BTBR mice compared to vehicle-treated group (Fig. 5C, P<0.05).

Upregulation of chemokine CCL2 and activation of microglia can induce pro-inflammatory cytokines, such as IL-1 β , IL-6, and CXCL10 (11,24). To analyse the effect of HJ on the mRNA expression of pro-inflammatory cytokines, the mRNA expression levels of IL-1 β , IL-6, and CXCL10 were measured in the hippocampi of the mice. Interestingly, the mRNA expression of IL-1 β and IL-6 were significantly decreased by HJ treatment (Fig. 5D, P<0.05). The mRNA expression of CXCL10 also showed a decreasing trend in the HJ-treated group, although it did not reach statistical significance (Fig. 5D, P=0.086).

NR2B and CaMKII α signalling is decreased by HJ treatment in the hippocampus of BTBR mice. To investigate the effects of HJ on the NR2B subunit of NMDA receptors and CaMKII α signalling, western blot analysis was performed in the hippocampus of vehicle-treated and HJ-treated mice. In the hippocampus of BTBR mice, the phosphorylation of NR2B and CaMKII α was significantly increased compared to their hippocampal expression in B6 mice (Fig. 6A and B). The

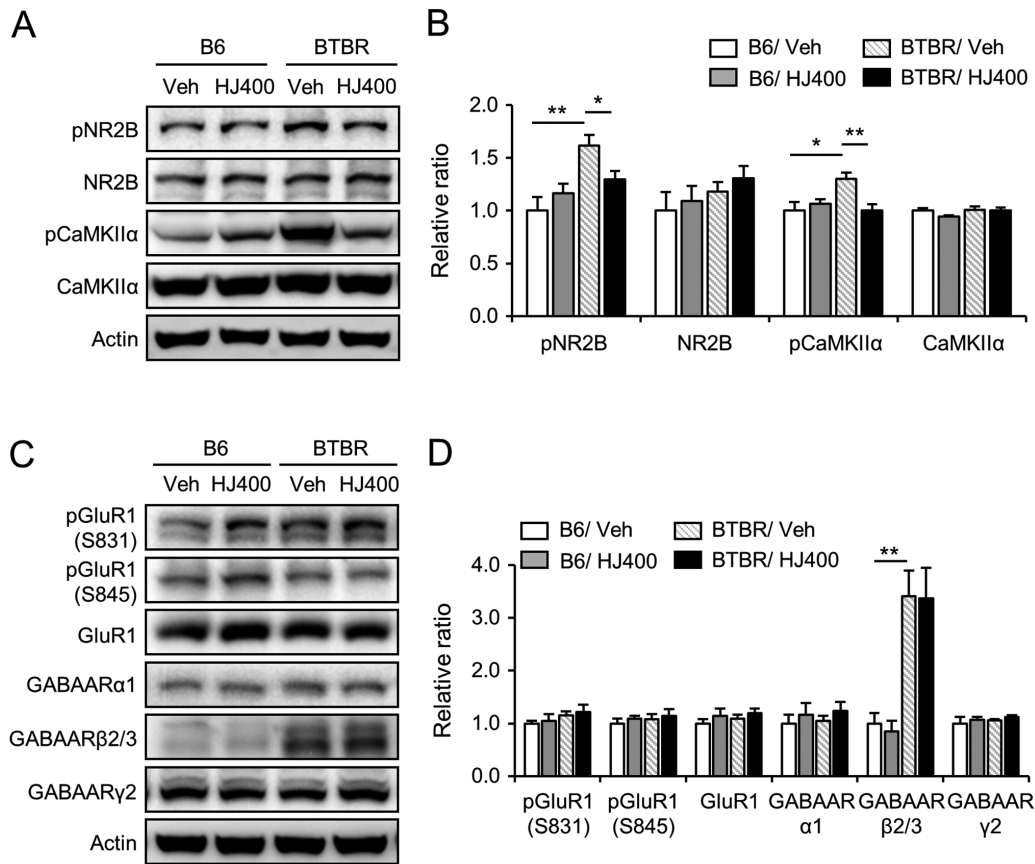


Figure 6. Effects of HJ treatment on the phosphorylation of NR2B and CaMKII α in the hippocampus of C57BL/6J and BTBR mice. Western blot analysis of NMDA receptor subunit NR2B, pNR2B (Tyr1472), CaMKII α , pCaMKII α , AMPA receptor subunit GluR1, pGluR1 (Ser831), pGluR1 (Ser845), and GABA receptor subunits α 1, β 2/3, and γ 2 in the hippocampus of the vehicle-treated (vehicle) and 400 mg/kg HJ-treated (HJ400) B6 or BTBR mice. Band densitometry values normalised to actin levels. (A) Representative image and (B) quantitative analysis of NMDA receptor subunit NR2B, pNR2B (Tyr1472), CaMKII α , pCaMKII α (Thr286) in the hippocampus of the vehicle-treated (vehicle) and 400 mg/kg HJ-treated (HJ400) B6 or BTBR mice. (C) Representative image and (D) quantitative analysis of AMPA receptor subunit GluR1, pGluR1 (Ser831), pGluR1 (Ser845), and GABA receptor subunits α 1, β 2/3, and γ 2 in the hippocampus of the vehicle-treated (vehicle) and 400 mg/kg HJ-treated (HJ400) B6 or BTBR mice. * $P < 0.05$ and ** $P < 0.01$ vs. indicated group. Data are presented as mean \pm SEM. HJ, *Humulus japonicus*; NR2B, N-methyl-D-aspartate receptor subtype 2B; CaMKII α , calcium/calmodulin-dependent protein kinase type II subunit α ; p, phosphorylated.

administration of HJ significantly decreased phosphorylation of NR2B and CaMKII α in the hippocampus of BTBR mice (Fig. 6A and B), but not in B6 mice. Taken together, these data indicate that HJ attenuates the NR2B and CaMKII α -mediated signalling pathways in the hippocampus of BTBR mice. However, the protein expression and phosphorylation (at Ser831 and Ser845) of the GluR1 subunit of AMPA receptor were not altered by HJ treatment (Fig. 6C and D).

In addition, we used western blotting to test whether HJ treatment affects the protein expression of GABA receptors. The expression of GABA receptor subunits α 1, β 2/3, and γ 2 was analysed in the hippocampus of vehicle-treated and HJ-treated B6 or BTBR mice. The protein expression of GABA receptor subunit β 2/3 was enhanced in the hippocampus of BTBR mice compared to B6 mice (Fig. 6C and D, $P < 0.01$). However, the protein expression of GABA receptor subunits α 1, β 2/3, and γ 2 was not regulated by HJ treatment in BTBR and B6 mice.

Discussion

ASD is a neurodevelopmental disorder characterised by deficits in social interaction and restrictive, repetitive, and stereotypical patterns of behaviour. However, there is no

pharmacological drug currently used to target these core ASD symptoms. In this study, we demonstrated the protective effects of HJ on autistic-like behaviours in BTBR mice. BTBR mice showed hyperlocomotion activity, more repetitive behaviours, lower sociability, and impaired cognitive function compared to B6 control mice. The administration of HJ in BTBR mice markedly decreased core autistic symptoms, such as self-grooming behaviour and lower sociability. Self-grooming behaviour represents a stereotypical pattern of behaviour in rodents (25). Self-grooming behaviour was significantly reduced by 400 mg/kg HJ treatment in BTBR mice, but not in B6 control mice. Moreover, we also found that sniffing and direct contact between the two mice were markedly increased by 400 mg/kg HJ treatment in BTBR mice, but not in control B6 mice. Although intellectual disability is not a DSM-5-specified criterion, BTBR mice showed significant impairments in novel object recognition, which markedly improved following HJ administration in BTBR mice.

Previous evidence has indicated the involvement of CCL2 in various neurological disorders, such as AD, epilepsy, and stroke (26). In addition, elevated CCL2 levels in cerebrospinal fluid and serum have a positive correlation with higher cognitive impairment (23). In an *in vitro* assay, CCL2 injured

neuronic dendrites in the CA1 region of hippocampal slices and induced primary hippocampal neuronic death (24,27). Treatment with 400 mg/kg HJ significantly decreased expression of CCL2 in the hippocampus. In order to explore the effect of HJ on neuroinflammation, we examined the mRNA expression of pro-inflammatory cytokines. Pro-inflammatory cytokines including IL-1 β , IL-6, and CXCL-10 were down-regulated by HJ administration in the hippocampus. CCL2 can significantly promote the expression of cytokines IL-1 β , IL-6, and CXCL-10 (24). In this study, the negative regulatory effect of HJ on CCL2 expression may influence the expression of these cytokines in the hippocampus.

Neuronic excitotoxicity is one of the major pathological mechanisms in various neurological disorders, including AD, depression, and ASD (28-30). Glutamate is the main excitatory neurotransmitter in the brain and plays a crucial role in synaptic transmission (30). However, excessive accumulation of glutamate results in neuronic excitotoxicity (31,32). Since CCL2 could enhance NMDAR-mediated excitatory postsynaptic currents and lead to hippocampal neuron death (27,33), we investigated the signalling pathway alteration mediated by NMDA receptors. Intriguingly, the phosphorylation levels of NR2B and CaMKII α were significantly reduced in the hippocampus of the HJ-treated group. In our study, although excessive accumulation of glutamate in BTBR mice has not been studied, pNR2B and pCaMKII α were markedly increased in the hippocampus of BTBR mice. Increased NR2B signalling was decreased by HJ treatment in BTBR mice. Thus, the effects of HJ on NR2B signalling may be beneficial in the hippocampus of BTBR mice.

Alterations of GABAergic signalling common in ASD patients have been detected in animal models of syndromic forms of autism and in BTBR mice (34). BTBR mice showed a reduced level of inhibitory neurotransmission mediated by GABA_A receptors in the hippocampus and enhancement of their inhibitory neurotransmission with positive allosteric modulators of GABA_A receptors ameliorated autism-like behaviours (34). In our study, the protein expression levels of GABA_A receptor subunits α 1, β 2/3, and γ 2 were analysed in the hippocampus of vehicle-treated and HJ-treated B6 or BTBR mice. Intriguingly, in the hippocampus, GABA_AR subunit β 2/3 was highly enhanced in the BTBR mice compared to B6 mice. However, there was no statistical difference in the expression of the GABA_AR β 2/3 proteins between vehicle- and HJ-treated groups. These data indicate that the protective effects of HJ on autistic-like behaviours are not caused by the modulation of the expression of GABA_A receptor subunits α 1, β 2/3 and γ 2.

In conclusion, this study showed that HJ treatment from postnatal 3-weeks to 9-weeks rescued repetitive behaviour and social interaction ability in BTBR mice. Further, we also explored the underlying mechanisms, including the influence of microglia activation, pro-inflammatory cytokines, and glutamate signalling. Overall, these data suggest that HJ treatment may provide a promising strategy to treat the core symptoms of ASD. The negative regulatory effects of HJ on NR2B signaling pathway and CCL2 expression may have a good influence in brain. The anti-autistic effects of HJ may last relatively long. The treatment-period studies are needed to evaluate the treatment strategy. In addition, our previous study on chemical profiling of HJ using HPLC-qTOF-MS and NMR

found biologically active substances from HJ against PD (35). Further studies are needed to evaluate the effects of these compounds on ASD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HYP, CHL and KSK designed the study; HYP, JG, YKR, DHC, JRN and JPA performed the experiments; HYP, JG, YKR, JRN, WKO, PLH and KSK analysed the data; and HYP, CHL and KSK interpreted data and wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Use and Care Committee of the Korea Research Institute of Bioscience and Biotechnology.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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