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Synthetic Cannabinoid-Induced Immunosuppression Augments Cerebellar Dysfunction in Tetanus-Toxin Treated Mice

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Abstract

Synthetic cannabinoids are one of most abused new psychoactive substances. The recreational use of abused drug has aroused serious concerns about the consequences of these drugs on infection. However, the effects of synthetic cannabinoid on resistance to tetanus toxin are not fully understood yet. In the present study, we aimed to determine if the administration of synthetic cannabinoids increase the susceptibility to tetanus toxin-induced motor behavioral deficit and functional changes in cerebellar neurons in mice. Furthermore, we measured T lymphocytes marker levels, such as CD8 and CD4 which against tetanus toxin. JWH-210 administration decreased expression levels of T cell activators including cluster of differentiation (CD) 3ε, CD3γ, CD74p31, and CD74p41. In addition, we demonstrated that JWH-210 induced motor impairment and decrement of vesicle-associated membrane proteins 2 levels in the cerebellum of mice treated with tetanus toxin. Furthermore, cerebellar glutamatergic neuronal homeostasis was hampered by JWH-210 administration, as evidenced by increased glutamate concentration levels in the cerebellum. These results suggest that JWH-210 may increase the vulnerability to tetanus toxin via the regulation of immune function.

Key Words: New psychoactive substances, Cytokine, T cell activator, Tetanus toxin, Motor impairment, Glutamate

INTRODUCTION

New psychoactive substances (NPS) have adverse cardiovascular, neurological, gastrointestinal, and pulmonary effects. However, NPS have in general been poorly characterized. Most available data on NPS-induced toxicity are derived from retro- or prospectively analyzed cases of intoxication as well as interviews with drug users, and are therefore of limited scientific value (Hohmann *et al*., 2014). Preclinical studies are required to evaluate toxicity; however, most studies have focused on the dependence potential and neuropsychiatric effects of NPS.

Synthetic cannabinoids are one of most frequently abused NPS and are associated with a risk for dependence that is similar to that of natural and botanical compounds. There are several hundred cannabinoid agonists that can potentially be abused with variable affinity for cannabinoid receptor type 1 (CB1) and CB2 (Fattore and Fratta, 2011). The endocannabinoid system regulates physiological processes such as caloric balance and the control of arterial smooth muscle tone (Hohmann *et al*., 2014). CB1 receptors are mainly found in the nervous system and are expressed by particular types of neurons (Seely *et al*., 2011). Synthetic cannabinoids are potent CB1 agonists that exert delta-9-tetrahydrocannabinol (THC)-like effects, with include alterations in mood, perception, sleep, and wakefulness, body temperature, and cardiovascular function (Hermanns-Clausen *et al*., 2013). However, their side effects are more varied and severe than those of THC, with the more common ones being tachycardia, arterial hypertension, hyperglycemia, hypokalemia, hallucinations, and agitation (Hohmann *et al*., 2014).

Given the expression patterns of CBs in the immune system, it is presumed that cannabinoids regulate the immune response. Immune cells express high levels of CB2 mediating cannabinoid anti-inflammatory effects, immunomodulation, and immunosuppression (McKallip *et al*., 2002a, 2002b; Yao and Mackie, 2009; Rieder *et al*., 2010). Otherwise, CB1 is present in many immune cells at relatively low levels, and there are few instances in which CB1 was determined to mediate immune systems effects of cannabinoids (Berdyshev,

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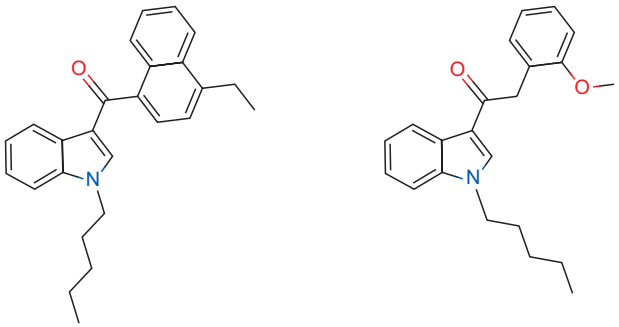

JWH-210 JWH-250

Fig. 1. Chemical structures of the sythetic cannabinoids JWH-210 and JWH-250.

2000). Cannabinoids were found to diminish resistance to infection in humans and animals (Friedman *et al*., 2003) and drug abuse is commonly reported among persons with tetanus (Pascual *et al*., 2003). Tetanus toxin (TeNT) is a disulphide-linked heterodimeric protein toxin responsible for the spastic paralysis characteristic of tetanus infection. TeNT has been reported to reduce glutamatergic neurotransmission in the cerebellum and to induce motor impairment (Kim *et al*., 2009; Yamamoto *et al*., 2003). The cleavage of vesicle-associated membrane proteins 2 (VAMP2) mediates TeNT-induced deficit in neurotransmission (Yeh *et al*., 2010; Blum *et al*., 2012). TeNT is processed by major histocompatibility complex class I and class II pathway and recognized by CD8+ or CD4+ T lymphocytes (immunology 2000, 100, 178-184). JWH-210 is a cannabimimetic alkylindole and a highly efficacious agonist at the CB1 and CB2 receptor with Ki values of 0.46 and 0.69 nM, respectively (Huffman *et al*., 2005a, 2005b). However, the detailed effects of JWH-210 on the immune response to tetanus infection are not well understood. To address this issue, in the present study we investigated the effects of the synthetic cannabinoids JWH-210 on immune function which is associated with TeNT clearance. We also measured T cell activator and cytokine levels in primary cultured splenocytes in mice following treatments of JWH-210. Furthermore, we aimed to determine if the administration of synthetic cannabinoids induced motor behavioral deficit and functional changes in cerebellar neuronal transmission in mice injected with TeNT.

MATERIALS AND METHODS

Animals

Seven-week-old male ICR mice were obtained from NIFDS animal supply facility, with Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC, Frederick, MD, USA) full accreditation, and were housed in a temperature-controlled room at 22 ± 2°C with a 12-hour light/dark cycle (light on 08:00 to 20:00) and were provided a solid diet and tap water ad libitum for 1 week. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Ministry of Food and Drug Safety (MFDS; Cheongju, Korea).

Materials

JWH-210 and JWH-250 (Fig. 1) were purchased from Cayman Chemical (Ann Arbor, MI, USA). TeNT (as a national reference standard) was obtained from the National Institute of Food and Drug Safety Evaluation (NIFDS), Ministry of Food and Drug Safety. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Fixed bar test

JWH-210 or JWH-250 (0.1 mg/kg, i.p.) was administered to mice at 14:00 for 5 days. TeNT (20 ng/mouse, i.c.v.) was injected into the right ventricle 24 h after the last administration of JWH-210 or JWH-250. Fixed bar test was performed for three consecutive days according to the previous report (Yamamoto *et al*., 2003), with scoring the performance of mice as follows: 1-5 sec=1, 6-10 sec=2, 11-20=3, 21-30 sec=4, >30 sec=5. Immediately after last fixed bar test, the animals were decapitated and the cerebellum was collected for further studies.

Immunoblot assay

Cerebellum tissues were homogenized by sonication in RIPA buffer containing protease inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). The homogenates of cerebellum were subjected to SDS-PAGE (4-15%, Bio-Rad, Hercules, CA, USA), and immunoblotting was performed. After blocking, membranes were incubated overnight at 4°C with anti-VAMP2 (rabbit, 1:1,000, Novus Biologicals, Littleton, CO, USA), anti-SV2 (mouse, 1:1,000, DSHB, Iowa City, IA, USA), anti-CB1R (rabbit, 1:1,000, Abcam, Cambridge, MA, USA), anti-mGluR1a (rabbit, 1:2,000, Sigma-Aldrich), anti-Iba1 (goat, 1:500, Abcam), or anti-Tuj1 (mouse, 1:40,000, Sigma-Aldrich) antibodies. Horseradish peroxidase-conjugated anti-rabbit (1:3,000, Sigma-Aldrich), anti-goat (1:1,000, Sigma-Aldrich), or anti-mouse (1:2,000, Sigma-Aldrich) antibodies were added for 1 hour at room temperature, and the immunoreactivity was visualized using an ECL Plus detection system (GE Healthcare, Piscataway, NJ, USA).

Glutamate measurements

Cerebellum tissues were homogenized by sonication in 0.2 M perchloric acid (100 μM EDTA•2Na) and centrifuged at 20.000×*g* for 15 min. Supernatants were kept frozen until analysis. Tissue glutamate concentrations were measured by enzyme-linked immunoassay (ELISA). Ninety-six well plates were coated with cerebellum homogenates (10 μg/well) at 4°C overnight. After washing with PBS (phosphate buffered saline, 0.05% Tween 20), in each well, anti-glutamate antibody was added (rabbit, 1:1,000, ab37070, Abcam) and incubated for 2 hours at room temperature. Thereafter, the samples were treated with horseradish peroxidase-conjugated anti-rabbit antibody (1:1,000, Sigma-Aldrich) for 2 hours at room temperature and with substrate solution (R&D systems, Minneapolis, MN, USA) for 20 min. The optical density was measured at 450 nm with a micro-plate reader (SpectraMAX M5, molecular device, Sunnyvale, CA, USA) after stopping peroxidase response with a stop solution (R&D systems).

Cytokine measurements

Tissue interleukin 2 (IL-2) and interferon-gamma (IFN-γ) concentrations were measured using a DuoSet ELISA development system (R&D systems) according to the manufactur-

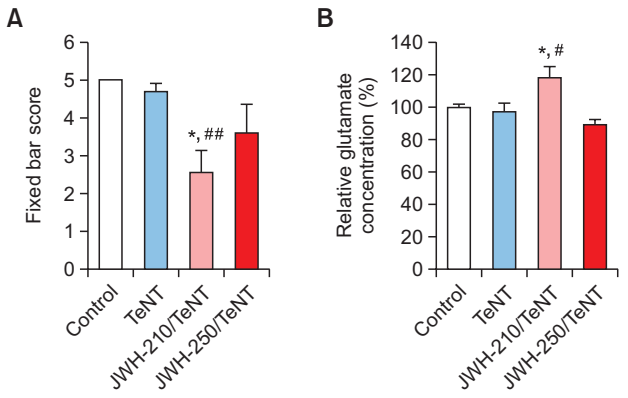

Fig. 2. Effects of synthetic cannabinoids on motor coordination of TeNT-treated mice and glutamate concentration in the cerebellum of TeNF-treated mice. JWH-210 (0.1 mg/kg) reduced the fixed bar scores of TeNT-treated mice (A) (n=8-13). ELISA revealed that JWH-210 (0.1 mg/kg) increased the glutamate levels of TeNT-treated mice (B) (n=4-8). *p<0.01 versus Control, $^{\#}p$<0.05 versus Tetanus, $^{\#\#}p$<0.01 versus Tetanus (One-way ANOVA followed by Bonferroni's test). Values indicate mean ± SE (n=8-13).

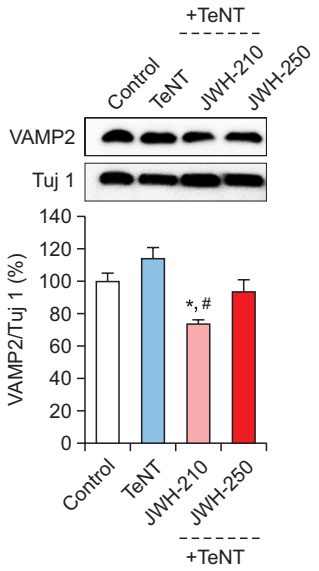

Fig. 3. Effects of synthetic cannabinoids on the VAMP2 expression levels in the cerebellum of TeNT-treated mice. Immunoblot analysis revealed that JWH-210 (0.1 mg/kg) decreased VAMP2 expression levels. *p<0.05 versus Control, $^{\#}p$<0.05 versus Tetanus (One-way ANOVA followed by Bonferroni's test). Values indicate mean ± SE (n=4-8).

er's manual. The cerebellum homogenates were subjected to ELISA and optical density was measured at 450 nm by using a micro-plate reader (SpectraMAX M5, molecular device).

Splenocytes culture

The spleens were isolated from naïve mice and single-cell suspensions were prepared by gently crushing the tissue using a sterile glass slide. The cells were seeded in 96-well plates (5×10^5 cells/well) with 100 μL Roswell Park Memorial Institute medium (RPMI 1640, Gibco, Waltham, MA, USA) supplemented with β-mercaptoethanol (50 μM), HEPES (10 mM), fetal bovine serum (5%), L-glutamine (1 mM), and antibiotics/antimycotics (Invitrogen, Carlsbad, CA, USA) and incubated for 6 h in 95% air/5% CO_2. Splenocytes were collected after treatment of JWH-210 (10 μM, 16 hour).

Quantitative real time reverse transcription (RT)-PCR

Complementary DNA of striatum and splenocytes was synthesized from total isolated RNA by using a SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). Subsequent quantitative real-time PCR was performed using the iCycler iQ5 real-time detection system (Bio-Rad) by using the SYBR Green I Master Mix (Thermo Fisher Scientific) detection format with an initial incubation of 50°C for 2 min. followed by 95°C for 15 sec. and 60°C for 1 min. cDNA was included in a 25-μL volume PCR reaction with following components: 0.125 μL each of forward and reverse primer that were purchased from Bioneer (Seoul, Korea, F: GGTATACGCCACGCTGAAGG, R: TAGCCACAGTACCGTTCCAGA for tyrosine hydroxylase; F: TGTCAAGCTCATTTCCTGGTATGA, R: CCTACTCCTTGGAGGCCATGTAG for GAPDH; F: CGTCCGCCATCTTGGTAGAG, R: ATTCAATGTTCTCGGCATCGT for CD3ε; F: TGGAGAAGCAAAGAGACTGACA, R: GCCATCCACTTGTACCAAATTC for CD3γ; F: ACCGAGGCTCCACCTAAAGAG, R: TTGACCCAGTTCCTGCCTG for CD74p31; F: TTCCTCACACCAAGAGCCG, R: TGTCCAGTGGCTCACTGCAG for CD74p41; N-4003 for CD4; N-4004 for CD8; N-4008 for IL-1α; N-4009 for IL-1β; N-4011 for IL-3; N-4012 for IL-5; N-4013 for IL-6; N-4014 for IL-10; N-4015 for TNFα; N-4018 TNFβ), 12.5-μL SYBR green, and 0.5 μg of cDNA with sterilized water. For the calculation of relative quantification, the $2^{-\Delta\Delta CT}$ formula was used, where: $-\Delta\Delta CT=(C_{T,target}-C_{T,GAPDH})$ experimental sample$-(C_{T,target}-C_{T,GAPDH})$ control sample.

Data analysis

Data represent the mean ± SE. Differences with respect to the vehicle-treated group were evaluated with the Student's t test or by One-way ANOVA, followed by the Bonferroni correction for equal variance or Dunnett's rank test for non-equal variance data using SigmaPlot v.13 software (SPSS Inc., Chicago, IL, USA). p<0.05 was considered statistically significant.

RESULTS

Effects of synthetic cannabinoids on motor coordination

We performed a fixed bar test by using a narrow wooden bar. The control, JWH-210/Vehicle, and JWH-250/Vehicle treated mice could stand easily on the narrow bar (Fig. 2A and Supplementary Fig. 1). The dosage of 20 ng of TeNT (i.c.v.) showed no significant effects on motor coordination itself. However, the JWH-210/TeNT-treated mice were unable to stand and crawled along the bar by grasping and pulling with their forepaws and dragging their hindlimbs. Furthermore, these mice fell off the bar sooner than those in the control and TeNT groups (p<0.05, Fig. 2A). In contrast, JWH-250/TeNT-treated mice did not show significant motor discoordination (Fig. 2A).

Effects of synthetic cannabinoids on glutamate concentration

To investigate the relationship between deficit in motor coordination and impaired cerebellar synaptic plasticity, we

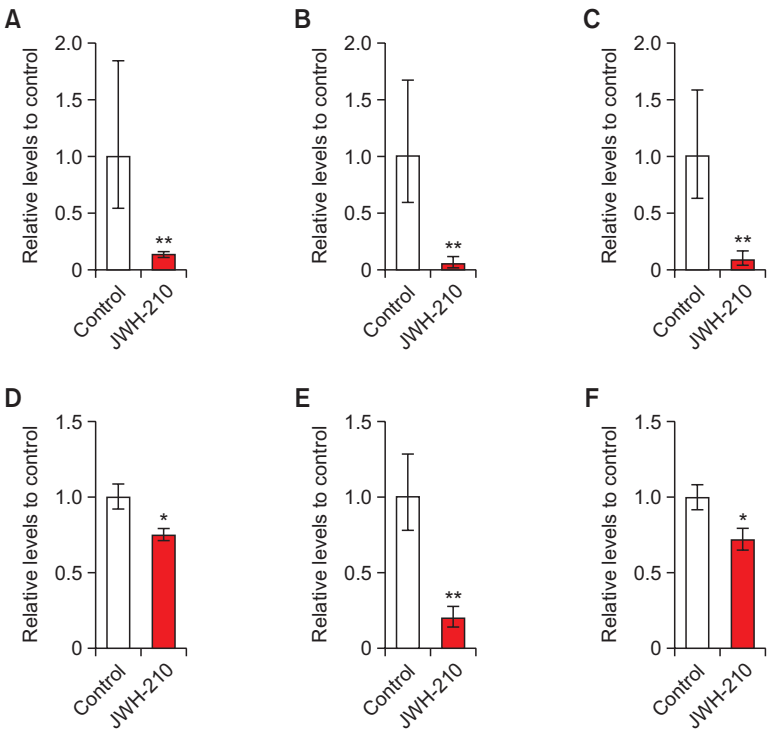

Fig. 4. Effect of JWH-210 on the mRNA expression of T-cell activators, T-cell markers and cytokines in splenocytes. JWH-210 reduced transcript levels of (A) CD3ε, (B) CD74p41, (C) CD74p31, (D) CD8, (E) IL-1β, and (F) IL-6. *p<0.05, **p<0.01 versus Control (Student's t test). Values indicate mean ± SE (n=6).

measured glutamate levels in cerebellum. The glutamate level of control group was 25.52 ± 0.51 μmol/g. JWH-210/TeNT increased glutamate levels in cerebellar tissue in comparison with the level in the control and tetanus groups, however JWH-250/TeNT did not show significant effects (Fig. 2B).

Effects of synthetic cannabinoids on expression of VAMP2, mGluR1a, SV2, and CB1R

TeNT-induced VAMP2 disruptions in cerebellum play a role in motor impairments (Yamamoto *et al*., 2003). We aimed to determine if synthetic cannabinoid treatment exacerbates TeNT-induced VAMP2 decrease in the cerebellum. We revealed that the expression levels of VAMP2 reduced in JWH-210/TeNT mice. However, tetanus and JWH-250/TeNT did not decrease VAMP2 expression levels significantly (Fig. 3). CB1R downregulation is also associated with the cerebellar dysfunction induced by delta9-tetrahydrocannabinol (Cutando *et al*., 2013). mGluR1a mediates cannabinoid signaling, and SV2 is a neuronal binding site of TeNT. However, the expression levels of mGluR1a, SV2, and CB1R did not change in this study (Supplementary Fig. 2).

Effects of synthetic cannabinoids on microglial activation

To clarify if synthetic cannabinoids evoke neuroinflammation in the cerebellum, we measured ionized calcium binding adaptor molecule 1 (Iba1) and cytokines expression levels, which are associated with microglial activation. However, tissue levels of Iba1, IL-2, and IFN-γ did not change in all groups (Supplementary Fig. 3).

Effects of JWH-210 on T cell activators and T cell markers in splenocytes

To clarify a possible mechanism underlying JWH-210-induced vulnerability to TeNT in mice, the effects of JWH-210 on the immune system was investigated. Quantitative RT-PCR experiments revealed that JWH-210 treatments (10 μM) reduced cluster of differentiation 3 antigen epsilon polypeptide (CD3ε), CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) p31, and CD74p41 in splenocytes (Fig. 4). Furthermore, JWH-210 reduced CD8, interleukin (IL)-1β, and IL-6 mRNA levels (Fig. 4) but had no effect on CD3 antigen gamma polypeptide (CD3γ), tumor necrosis factor (TNF) α and β, CD4, IL-1α, IL-5, and IL-10 expression (data not shown), suggesting that this synthetic cannabinoid is immunomodulatory and may cause increased susceptibility to TeNT.

DISCUSSION

Synthetic cannabinoids is one of most abused novel psychoactive substances. Lack of information on the toxicity and pharmacological activity of synthetic cannabinoids may mislead people to abuse substances without concerns of health risks including suppression of host resistance to infections. In this study, we aimed to determine if JWH-210 and JWH-250 induce susceptibility to TeNT in mice. A synthetic cannabinoid, JWH-210 (0.1 mg/kg, 5 days) induced motor impairments in TeNT-treated mice. The motor deficit is mainly associated with cannabinoid receptor activation, because only JWH-210 with the greatest binding affinity treatments showed reduced hold-

ing performance on the fixed bar test. We also showed that the glutamate concentration in the cerebellar tissue of JWH-210/TeNT mice increased. This upregulation of glutamate levels in the cerebellum may be because of the increase in intracellular, and not extracellular, glutamate (Julio-Pieper *et al*., 2011). According to Yamamoto *et al*. (2003), TeNT reduces glutamate release from the cerebellum, which contributes to deficit in motor coordination. An overall change of glutamate level in cerebellum is related to motor ataxia (Kim *et al*., 2003) and CB1R activation reduces neurotransmitter release (Hoffman *et al*., 2010). Therefore, although, we did not measure glutamate release in JWH-210/TeNT, we can assume that JWH-210/TeNT reduced the glutamate release, and consequently induced the increase in intracellular glutamate levels, which may compensate the deficit of glutamatergic neurotransmission. TeNT is a metalloproteinase and cleaves VAMP2, which is associated with the reduction of glutamate release in the cerebellum.We showed that the expression levels of VAMP2 in JWH-210/TeNT mice significantly decreased and holding times on fixed bar; however, the levels of mGluR1a and CB1R were not affected. JWH-210 also did not affect the expression of SV2, which is a receptor of TeNT in neurons. In addition, we excluded a possible role of neuroinflammation in JWH-210-induced cerebellar dysfunction, because the expression levels of Iba1 and microglial activation-related cytokines, such as IL-2 and IFN-γ, were not upregulated in the cerebellum of JWH-210/TeNT mice. Although, JWH-210 administration induced decreased VAMP2 expression levels in TeNT-treated mice, the exact mechanism underlying JWH-210-induced susceptibility to TeNT is not clear. However, we demonstrated that JWH-210 treatments resulted in the downregulation of T-cell activators such as CD3ε, CD74p41, and CD74p31 in splenocytes. CD3ε forms the T cell receptor-CD3 complex that is essential for T-cell development and the immune response (Gagnon *et al*., 2012; Brazin *et al*., 2014), while CD74 is a nonpolymorphic type II integral membrane protein that functions mainly as an major histocompatibility complex class II chaperone and has two different isoforms, namely p31 and p41 (Starlets *et al*., 2006). JWH-210 also inhibited the expression of CD8, a marker of helper T lymphocytes, which recognize TeNT (Kerblat *et al*., 2000), in accordance with *in vivo* experiment results (in submission data). Furthermore, JWH-210 decreased the levels of IL-1β and IL-6 in splenocytes. Immune cell density and cytokine gene profiles can be accurately determined by quantitative RT-PCR (Vremec *et al*., 2000; Mocellin *et al*., 2003; Tanaka *et al*., 2004). Cannabinoids have been shown to suppress T-cell proliferation and cytokine production in mouse spleen cells (Robinson *et al*., 2013, 2015). JWH-210 is a potent cannabinoid agonist at both the CB1 and CB2 receptors. Immune cells express high levels of CB2, which has anti-inflammatory, immunomodulatory, and immunosuppressive effects (McKallip *et al*., 2002a, 2002b; Yao and Mackie, 2009; Rieder *et al*., 2010). Therefore, we assume that JWH-210 has effects on immune system via CB2 receptors, although spleen expresses CB1 receptors (Supplementary Fig. 4). Together, these results suggest that JWH-210 increases a vulnerability to TeNT-induced motor impairments via the downregulation of immune functions.

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