Lithium Attenuates Cannabinoid-Induced Dependence in the Animal Model: Involvement of Phosphorylated ERK1/2 and GSK-3β Signaling Pathways

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Abstract- Cannabis is one of the most banned drugs in the world. Cannabinoid-induced dependence or withdrawal signs are indicated by the result of complex molecular mechanisms including upstream protein kinases (PKs), such as an extracellular signal regulated kinase1/2 (ERK1/2) and downstream glycogen synthase kinase-3β (GSK-3β), which lead to neuronal plasticity. In this study, we examined the protective effect of lithium (Li) as a potent ERK1/2 and GSK-3β modulator to prevent the development of dependence on cannabinoids. For this purpose, rats were treated twice daily with increasing doses of WIN 55,212-2 (WIN, 2-8 mg/kg, intraperitoneally (i.p.), for five consecutive days. AM251 (AM, 2 mg/kg), a cannabinoid antagonist, was injected i.p to induce manifestations of abstinence in rat dependency on WIN, and the subsequent withdrawal signs were recorded. To evaluate the preventive effect of Li, the rats were pre-treated with Li (10 mg/kg, i.p.) twice daily, 30 minutes before every injection of WIN. SL327, as an ERK1/2 inhibitor, was also injected (SL, 50 mg/kg, i.p.) 30 minutes before the last doses of WIN in separate groups. The p-ERK1/2, total ERK1/2, p-GSK-3β and total GSK-3β expressions were determined with Western blot method after 60 minutes, prior to the Li, WIN or AM injections. Li and SL pre-treatment attenuated the global withdrawal signs in regarding their modulation effect on the up-regulation of p-ERK1/2 cascade enhanced by AM injection. Furthermore, the p-GSK-3β expression was up-regulated with SL and Li pre-treatment against AM injection, without alteration on the total contents of ERK1/2 and GSK-3β level. Therefore, p-ERK1/2 and p-GSK-3β pathways are involved in the cannabinoid-induced dependence. However, no crosstalk was indicated between these two pathways. In conclusion, Li neuroprotectionwith regard to cannabinoid abstinence may occur through the regulation of the p-ERK1/2 cascade inconsequent of p-GSK-3β signaling pathways in rats.

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Introduction

Cannabinoids are the active components of *Cannabis sativa* (marijuana). Chronic consumption can cause milder withdrawal signs in comparison to opioids (1-3). Recent studies have reportedthe relation of opioids to cannabinoids' systems and, therefore, both systems have similar responses including anti-nociception, hypo-locomotion and hypothermia, as well as the development of cannabinoid-induced dependence or withdrawal signs (4). Cannabinoid withdrawal can be enhanced in animals by administrating AM251 or SR141716A (rimonabant) as cannabinoid receptor antagonists to further induce withdrawal signs (5,6). Cannabinoid ssuch as WIN 55,212-2 and tetrahydrocannabinol (THC) were also wildly used for induction of cannabinoid dependence in animal models (5,7). However, molecular mechanisms or signaling pathways regarding the development of cannabinoid-induced dependence or withdrawal signs are less clear.

The development of dependence or tolerance to cannabinoids may be mediated by CB1 receptors, a G-

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protein-coupled receptor (GPCR) and with theinteraction to Gi/0 proteins. This causes inhibition of adenylcyclase (AC) activity, changes in Ca^{2+} and K^{+} currents, activation of protein kinases (PKs), such as extracellular signal-regulated kinases (ERK), as well as focal adhesion kinase (FAK) and phosphatidylinositol-3-kinase (PI3K) (8).

Other studies have indicated that CB1 receptors mediated ERK activation through dopaminergic and glutamatergic mechanisms (9-12). Furthermore, SL327, a specific inhibitor of mitogen-activated protein kinase (MAPK) and the upstream kinase of ERK can prevent the development of tolerance to cannabinoid hypo-locomotion (13). GSK-3β is another downstream molecular mechanism related to cannabinoids, which is essentialin the induction of drug-induced withdrawal signs and has an important regulator of survival and apoptosis subject in neuronal cells (14,15). GSK-3β can interact with Bax, a protein of the intrinsic apoptotic cascade, which is involved in mitochondrial pore formation (16). GSK-3β is also related to JNK activation as apoptotic signaling (17). Therefore, upstream PKs such as the PI3K/Akt and ERK1/2 signaling pathways are capable of rescuing the neurons from induced apoptosis. However, the PI3K/Akt pathway is defined as the mainpathway to phosphorylation of GSK-3β to its in-activation state and to survival route in neurons (18, 19). Therefore, p-ERK1/2 and p-GSK-3β activation plays an important role in regulating cannabinoid abstinence. Li, as a potent inhibitor of the GSK-3β pathway and modulator of the ERK1/2 activation (20), inhibits cannabinoid tolerance (21), morphine-induced withdrawal signs and the development of physical dependence (22-24).

Aim of this study was to investigate the effect of Li pre-treatment on cannabinoid-induced dependence or withdrawal signs leading to neuronal plasticity in the animal model. The ERK1/2 and GSK-3β phosphorylation, as intrinsic molecular pathways related to cannabinoid-induced dependence or withdrawal signs, were studied. Authorsalso evaluated the crosstalk of p-ERK1/2 and p-GSK-3β signaling by administrating SL327 in cannabinoid-dependent rats.

After this study, the authors can state that Li pre-treatment can attenuate cannabinoid-induced dependence or withdrawal signs through the regulation of p-ERK1/2 and p-GSK-3β signaling pathways in animals; therefore, it will provide a potent pharmacotherapy target to overcome the cannabinoid-induced disorders.

Materials and Methods

Animals

Adult male Wistar rats, weighing 150-200 g, were obtained from Faculty of Pharmacy, Tehran University of Medical Sciences (TUMS). Animals were housed in groups of six per cage for one week under standard 12 h light/dark cycle at 22-24 ºC, and then were treated with approved Ethical Committee protocols for the use and care of laboratory animals of TUMS (25).

Induction of cannabinoid dependence

Rats were administrated interaperitonellay (i.p.) twice daily for five consecutive days with increasing doses of WIN (Day 1: 2 and 2 mg/kg; Day 2: 2 and 4 mg/kg; Day 3: 4 and 4 mg/kg; Day 4: 8 and 8 mg/kg; Day 5: 8 mg/kg) following the standard protocol (13, 26).

Expression of withdrawal signs

Two hours after the last dose of WIN (2-8 mg/kg, i.p); rats were placed in a circular clear plastic observation area for a 15-minute period of habituation. Then the animals were immediately observed for 15 minutes, followed by injections of AM251 (AM, 2 mg/kg, i.p.). The rats were then observed for a 30 minutes. The global withdrawal signs, including body tremor, piloerection, mastication, genital licks, sniffing, paw tremors and wet-dog shakes were checked for in each rat during 30 minutes at 5-minute intervals, and then the level of severity was obtained as the composite score for each animal as indicatedwith the global withdrawal score ranging from 0% to 100% (4,5,26).

Treatment procedures

To evaluate the relation of p-GSK-3β and p-ERK1/2 expression in the cannabinoid-induced dependence or withdrawal signs, groups of rats were pre-treated with Li (10 mg/kg, i.p.), 30 minutes before every dose of WIN (2-8 mg/kg, i.p.), and SL (50 mg/kg, i.p.) was administered 30 minutes before the last dose of WIN following the studies (13,26).

Western blotting

Rats were anesthetized with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (25 mg/kg, i.p.), and sacrificed one hour after the end of the experimental period in separate groups. The cerebellum brain section of the rats were immediately removed and put on ice and then stored at -80ºC until used. Western blotting was performed as described (27). Briefly, cerebellum tissues were homogenized with a manual homogenizer and then

were incubated with 200 µl of lyses buffer, including 10 mmol/lTris pH= 8, 0.1% SDS, 1% NP40, 0.5 mmol/l DDT, 0.5% Na-deoxycholate, 0.5 mmol/l PMSF and 0.5 µg/ml leupeptinand, one complete protease inhibitor cocktail tablet per 10 ml of buffer (Roche), at 4ºC for 30 minute for the prevention of phospho-protein breaks by protease or phosphatase enzymes (28), and then the products were centrifuged at 14000 rpm in 4ºC for 5 minutes and the supernatant was removed. At this point, 20 µl of loading buffer, including 50 mmol/l Tris pH= 6.8, 2% SDS, 4% 2-mercaptoethanol, 10% glycerol and 0.1% bromophenol, was added to the product and boiled for 10 minutes. The samples were once again centrifuged in 14000 rpm for five minutes; equal amounts of supernatants (20 µl) were loaded to 10% SDS-PAGE, and then transferred to nitrocellulose membranes prepared from Amersham (GE Healthcare, UK).

Membranes were then immunoblot with anti-ERK1/2, anti-phospho-ERK1/2, anti-GSK-3β and anti-phospho-GSK-3β at 1: 1000 dilution from Cell signaling Technology Company (USA), or anti β-actin at 1: 2000 dilution as the internal control (CTRL), prepared from Santa Cruz Biotech (USA) at 4ºC, overnight. For thedetection of an antibody reaction, blots were incubated with horse-radish peroxidase (HRP)-conjugated secondary antibody at 1: 10000 dilutions for 60 minutes at room temperature. Detection was carried out using a chemiluminescence Western Blotting Kit (Roche; Mannheim, Germany) on X-ray film (Kodak; Rochester, NY). Immunoreactive bands were analyzed using ImageJ software (NIH; USA), normalized to β-actin, and then expressed as a percent of CTRL to mean ± SD of at least three independent experiments.

Statistical analysis

All results were presented as mean ± SD. The statistical analyses were performed using one-way Analysis of Variance (ANOVA) following the post-host test of Tukey. Differences with p-value less than 0.05 were considered statistically significant.

Results

Behavioral studies

Results of this study indicated that acute injection of AM (2 mg/kg, i.p) enhanced significant increases (P<0.001) of global withdrawal signsin dependent rats treated with increasing doses of WIN (2-8 mg/kg, i.p) as compared to CTRL (Figure 1). However, pre-treatment with Li can significantly reduce (P<0.01) these withdrawal signs as compared to the AM+WIN group. Furthermore, acute pre-treatment of rats with SL (50 mg/kg, i.p.) significantly ameliorated the abstinence signsinduced by AM injection, as compared to the AM+WIN group. It was indicated that the potential effect of Li and SL was to prevent the cannabinoid-induced withdrawal in animals, and, therefore, it was suggested that ERK1/2 phosphorylation was required for excitation induced by cannabinoid receptor antagonists in animals. Therefore, its in-activation may be one of the protective intrinsic mechanisms of Li against neuronal plasticity induced by cannabinoid abstinence.

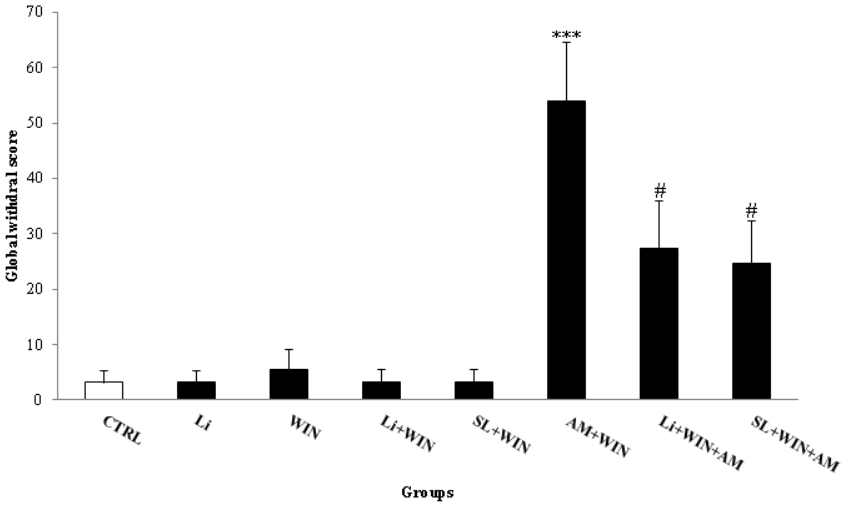

Figure 1. Somatic expression of cannabinoid withdrawal enhanced by acute administration of AM251 (AM) in rats dependent upon WIN 55, 212, 2 (WIN). Rats were treated twice daily with increasing doses of cannabimimeticamino-alkyl-indole, WIN 55, 212, 2 (WIN, 2-8 mg/kg, i.p., n = 6) for five consecutive days, and more than two hours after the last dose of WIN, the cannabinoid antagonist AM was acutely injected (2 mg/kg, i.p., n = 6). All data is presented as mean ± SD. *P<0.05; **P<0.001; ***P<0.0001 compared to control (CTRL). $^{\#}P$<0.05 compared to AM+WIN group.

Molecular evaluations

To determine the possible molecular mechanisms related to cannabinoid withdrawal signs, the authors removed the cerebellum of the experimental rats after the end of experiment period, and the expressions of p-ERK1/2, total ERK1/2, p-GSK-3β and total GSK-3β proteins were analyzed by the Western blotting method (Figure 2A). Treatment of rats with WIN significantly down-regulated the p-ERK1/2 and up-regulated the p-GSK-3β expressions in the cerebellum (P=0.041 and P<0.01), respectively, as compared to CTRL (Figure 2 B & C). Furthermore, no significant change was indicated with pre-treatment of Li on the p-ERK1/2 and p-GSK-3β expressions in the cerebellum as compared to CTRL (Figure 2 B & C). Furthermore, Li pre-treated to WIN indicates no significant change on the expression of p-ERK1/2; however, Li pre-treated to WIN significantly (P<0.001) up-regulated the expression of p-GSK-3β, as compared to CTRL (Figure 2C). AM injection significantly (P<0.001) up-regulated the p-ERK1/2 and down-regulated the p-GSK-3β expression in cannabinoid dependent rats, as compared to CTRL (Figures 2 B and C). This data indicated that p-ERK1/2 and p-GSK-3β signaling pathways were important in the cannabinoid-induced dependence or withdrawal signs in rats.

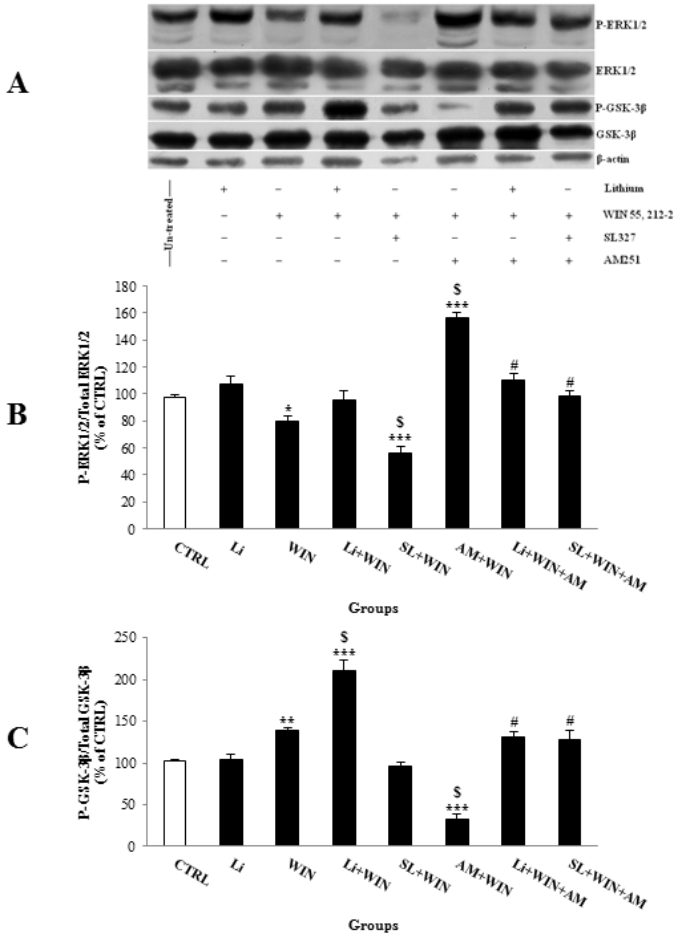

Figure 2. The effect of the cannabinoid receptor antagonist AM251 on WIN 55, 212-2-included changes in phosphorylated-ERK1/2 (p-ERK1/2), phosphorylated-GSK-3β (p-GSK-3β) and total amount of ERK1/2 and GSK-3β in the rat cerebellum. The animals were treated twice daily with increasing doses of the cannabinoid antagonist WIN 55, 212-2 (WIN, 2-8 mg/kg, i.p., n = 6) for five consecutive days, and more than two hours after the last dose of WIN, the cannabinoid antagonist AM was injected (2 mg/kg, i.p., n = 6). Rats were pre-treated with lithium (Li, 10 mg/kg., i.p., n = 6) twice daily, 30 minutes before each dose of WIN. Rats were also pre-treated with an acute dose of SL327 (SL, 50 mg/kg, i.p., n= 6), as a selective ERK1/2 inhibitor, 30 minutes before the last dose of WIN. Control (CTRL) rats received normal saline (NS, 1 ml/kg, i.p., n = 6). (A) Representative Western blots of p-ERK1/2, total ERK1/2, p-GSK-3β and total GSK-3β protein expressions; (B & C) the quantitative analysis of p-ERK1/2, total ERK1/2, p-GSK-3β and total GSK-3β. All data is presented as mean ± SD. *P<0.05; **P<0.001; ***P<0.0001 compared to control (CTRL). $^{\$}P$<0.05 compared to WIN group. $^{\#}P$<0.05 compared to AM+WIN group.

Lithium and SL327 regulates p-ERK1/2 and p-GSK-3β pathways

Pre-treatment of rats with Li (20 mg/kg/day, i.p.) and acute injection of SL (50 mg/kg, i.p.) prevented the significant (P<0.001) up-regulation of the p-ERK1/2, and also significantly (P<0.001) blocked the down-regulation of the p-GSK-3β expression by AM injection, as compared to the AM+WIN group (Figures 2 B & C). These results showedthat no crosstalk was between the p-ERK1/2 and p-GSK-3β pathway, and therefore more other PKs than ERK1/2 may be mediated the phosphorylation of GSK-3β pathway. Furthermore, no significant change was in the total amount of the ERK1/2 and GSK-3β expression in experimental groups, as compared to CTRL (Figure 2A), wherein no breakdown occurred in the ERK1/2 and GSK-3β proteins. Therefore, Li pre-treatment can attenuate cannabinoid abstinence enhanced by AM through regulation of p-ERK1/2 cascade inconsequent of the p-GSK-3β signaling pathway.

Discussion

In this study, Li, as a neuroprotective drug at a lowered dose (20 mg/kg/day, i.p), and pre-treatment experiments can attenuate thedevelopment of cannabinoid dependence or withdrawal signs enhanced by cannabinoid receptor antagonists in the animal model. Two molecular signaling pathways, including p-ERK1/2 and p-GSK-3β pathways were predominantly related to CB1 receptor, which were highly expressed in the cerebellum brain section (29). Then Li neuroprotective molecular mechanisms, including p-ERK1/2 and p-GSK-3β modulation, were evaluated in rats. Although cannabinoids were used for a long time for medicinal and recreational goals, new pharmacological effects, including their anti-cancer properties, was discovered recently (30,31). Cannabis disorders affect more adults in the world andnumber of its users have increased (32,33), but a few cannabis users do seek pharmacotherapy for their disorders.

The protective effect of Li against peripheral neuropathy induced by Vincristine (VCR) with the dose of 20 mg/kg/day i.p. was reportedin a previous study in animals (34). These Li neuroprotective effects have occurred at the sub-therapeutic concentrations without associationto its received therapeutic plasma concentration (34,35). Studies have reported the amelioration effect of acute and chronic administration of Li on the opioid-induced dependence or withdrawal signs (36,37). Opioids and other drugs, such as cocaine, have functional interaction with theendocannabinoid system/CB1 receptors to induce similar withdrawal signs and are insensitive to further concern in humans (4,38,39). Furthermore, the inhibitory effect of Li on cannabinoid tolerance was showedin vitro (21). However, the attenuating effect of Li on the cannabinoid-induced dependence or withdrawal signs are less clear in the animal model, and little is known about the possible molecular mechanisms or signaling pathways about the protective role of Li has been reported.

Therefore, we indicated that pre-treatment with Li (20 mg/kg/day, i.p) has a preventive effect on the cannabinoid withdrawal signs enhanced by acute injection of AM (2 mg/kg, i.p.) in cannabinoid dependent rats treated with increasing doses of WIN (2-8 mg/kg, i.p.). Studies have indicatedthe relation of dopaminergic (dopamine, D2 receptor), glutamatergic (glutamate, GLUT) and GABAnergic (Gamma-amino-butyric-acid, GABA) systems on the drug-induced withdrawal signs (36,40,41).

GLUT interacted with the NMDA/NO cascade, which has played an important role in the induction of cannabinoid-induced withdrawal, leading to neuronal plasticity and Ca2+/calmodulin clusters (42). Therefore, NMDA/NO inhibition was the other neuroprotective mechanism of Li introduced against excitation in the brain, and it has regulated the signaling pathway with ERK (43). It has also shown that D2 receptors regulated the cyclic adenosine mono phosphate (cAMP)-PKA (protein kinase A) and Ca^{2+} pathway by mediating GPCRs, wherein GPCR can function through a PKB (Akt)-GSK-3 signaling pathway (18) since, the cannabinoid withdrawal was underlying to calmodulin-stimulated AC and PKA activities in the cerebellum (44). Therefore, the cAMP/CREB/PKA mediating pathway is a major documented neuroprotective mechanism of Li against toxicants (45,46). ERK1/2 and GSK-3β activation have been shown to have a relationship with these systems (18) and. Therefore, they can regulate the expression of behavior alteration in cannabinoid abstinence induced in rats. WIN down-regulated the p-ERK1/2 (activation) cascade and up-regulated the p-GSK-3β expression (in-activation), in which the antagonist with AM injection was found. It has reportedthat activation of β-adrenergic receptors by isoproterenol mimics forskolin (FSK) to elicit a cAMP-dependent PKA inhibition of CB1 receptor functions, and it conceded the inhibitory effect of

cannabimimeticaminoalkylindole, WIN 55, 212-2 on the activation/phosphorylation of the ERK1/2 pathway against AM injection (40). Furthermore, the presynaptic inhibitory action of CB1 receptors at corticostriatal synapses could be negatively regulated by a cAMP/PKA-mediated receptor phosphorylation related to glutamatergic transmissions to induce withdrawal sign expressions (40). Therefore, ERK1/2 in-activation and GSK-3β induced phosphorylation (in-activation) were regulated with the activation of β-adrenergic and in-activation of glutamatergic/dopaminergic receptors, respectively (40). Therefore, both the p-ERK1/2 and p-GSK-3β were important in the cannabinoid-induced dependence or withdrawal signs.

Li is a putative inhibitor of the GSK-3β intrinsic pathway in which it can inhibit the GSK-3β activity by inducing its phosphorylation (19). It has been reported that GSK-3β phosphorylation is regulated mainly with the PI3K/Akt kinase pathway, and it was important to the actions of thedopaminergic system (18); however, the knowledge of the mediating effect of the other upstream PKs, such as ERK1/2 to induce cannabinoid-induced dependence or withdrawal signs is limited. Studies have demonstrated the important role of the upstream RAS/ERK1/2 cascade phosphorylation (activation) in the cannabinoid-induced tolerance; however, its crosstalk to the downstream pathway to induce cannabinoid abstinence has not yet cleared. However, it has indicated the crosstalk of NO and ERK1/2 signaling pathways to mediate opioid withdrawal signs in rats (47). The down-stream GSK-3β pathway can regulate the special gene expressions in nuclear neuronal cells that are important to induce dependence or withdrawal signs. To say that there is crosstalk and sequence relations of p-ERK1/2 to p-GSK-3β signaling pathway, it must down- or up-regulate p-ERK1/2 expressions consequently and respectively, with down- or up-regulation of the p-GSK-3β expression. Current study showed that Li and SL pre-treatment reduced the up-regulation of p-ERK1/2 and up-regulated the p-GSK-3β expression against acute administration of rats with AM injection. The authorsindicated that when p-ERK1/2 was down-regulated with WIN or SL and Li pre-treatment (SL+WIN or Li+WIN) in experimental groups, however, the p-GSK-3β expression was up-regulated in WIN or Li+WIN groups, and no significant change was indicated between the SL+WIN group as compared to CTRL. Furthermore, when the p-ERK1/2 level was up-regulated in the AM+WIN group, however, no significant increase was indicated in the expression of p-GSK-3β in the AM+WIN group, compared to CTRL. Therefore, no crosstalk occurred between these two pathways. This means that Li pre-treatment attenuates cannabinoid abstinence through the regulations of p-ERK1/2 cascadeinconsequent of p-GSK-3β pathway. Indeed, the PI3K/Akt pathway has a major up-stream kinase to regulate the GSK-3β phosphorylation. However, both the p-ERK1/2 and p-GSK-3β pathways were inconsequentially playing a central role in the development of cannabinoid dependence and induction of its withdrawal signs. Presentresults will provide a therapeutic molecular target to reduce the neuronal plasticity and suggests that Li neuroprotective mechanisms prevent the development of cannabinoid-induced dependence or withdrawal signs. Therefore, regulating the effect of Li on CB1 receptors and p-ERK1/2 and p-GSK-3 pathways is a potential strategy for preventing clinical manifestations, including pain, drug dependence, epilepsy, anxiety, depression, Parkinson's and Huntington's diseases, amyotrophic lateral sclerosis, stroke, cancer, glaucoma, autoimmune uveitis, osteoporosis, sepsis, renal, hepatic, intestinal and cardiovascular disorders induced by cannabinoids (48).

In conclusion, presentresults suggested the potential of Li pre-treatment for reducing cannabis disorders. Furthermore, the regulation of ERK1/2 and GSK-3β phosphorylation was an important mechanism to neuroprotective mechanism of Li on the development of cannabinoid-induced dependence or withdrawal signs in rats. However, no crosstalk between these two pathways is indicated. Therefore, a clinical trial study to include the Li as a maintenance therapy to prevent cannabinoid-induced disorders is recommended, and it may be the next step in this type of study in the future.

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