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Original Article

Induction of long-term potentiation at Schaffer collateral-CA1 synapses in mice hippocampus after IMPX977 administration

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ABSTRACT

Objective: To investigate the effects of IMPX977 on long term potentiation (LTP) at Schaffer collateral-CA1 synapses *in vitro* and on methyl CpG binding protein 2 (Mecp2) expression in mice cortex and hippocampus.

Methods: Thirty-two C57BL/6 mice were randomly divided into four groups: control, olive oil (vehicle), IMPX977 low (5 mg/kg) and high (15 mg/kg) groups. Mice were administrated every other day orally for two weeks. Extracellular recording technique *in vitro* was used to record the effects of IMPX977 on Schaffer collateral-CA1 LTP pathway in acute mice hippocampal slices. The Mecp2 protein expression level was detected by Western blotting.

Results: Compared to the control group, vehicle did not alter the synaptic transmission in Schaffer collateral-CA1 synapses, however, IMPX977 at concentrations of 5 mg/kg and 15 mg/kg significantly enhanced fEPSP (field excitatory postsynaptic potential) slope in Schaffer collateral-CA1 pathway to (179.6 ± 17.8)% and (191.4 ± 21.4)%, individually 60 min after HFS, IMPX977 improved LTP induction significantly at Schaffer collateral-CA1 pathway at least. Also, IMPX977 significantly elevated MeCP2 protein level in cortex.

Conclusion: The effects of IMPX977 on synaptic transmission and Mecp2 protein expression provided convincing evidence that IMPX977 could be promising new drug candidates for Rett syndrome treatment.

1. Introduction

Rett syndrome (RTT) is a progressive neurodevelopmental disorder that occurs almost exclusively in females (Stallworth et al., 2019; Wong et al., 2018). RTT is caused by mutations in the gene encoding methyl-CpG binding protein 2 (MeCP2), an epigenetic regulator of mRNA transcription (Kyle et al., 2018; Ip et al., 2018). IMPX977, code name of one of the constituents of cotton seeds, displays various drug properties, including antifertility, anticancer and etc., which is mainly used for subsidence of swelling, heat-clearing and detoxifying in traditional medicine and also has been used as a male contraceptive drug for many years. In the previously work, Hu et al. reported that MeCP2 is highly expressed in the cerebellum, cortex and hippocampus, less expressed in heart, spleen and lung; MeCP2 expression level was elevated after 2 weeks administration of IMPX977 in female rats (Hu et al., 2017). Although the molecular mechanism underlying the mutation-caused abnormal signs in RTT is not clear, targeting hypothesized that up-regulating the expression of MeCP2 protein may be conducive to relieve the symptoms of RTT disorder. Also, IMPX977 increased the expression of brain derived neurotrophic factor (BDNF) which is crucial for neuronal systems survival, development and the synaptic function (Zhao et al., 2019). Numerous studies showed that the increased BDNF expression would ameliorate Rett syndrome-like phenotype and prolong the lifespan of Mecp2 deletion animals (Downs et al., 2018; Tsai, 2016; Krishnan et al., 2015; Nissenkorn et al., 2017). In addition to diverse features at the genetic and molecular levels, the abundant expression in several regions of the central nervous system has implicated BDNF as a potent modulator in many aspects of neuronal development, as well as synaptic transmission and plasticity. It is quite interesting that the results from the RNA-sequencing analysis showed that the related biologic processes were enriched to neuron function, like synaptic transmission, dopaminergic

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calcium ion regulation and calcium ion concentration after IMPAX977 administration. Besides, the upregulated differentially expressed genes (DEGs) were mapped to cocaine addiction, morphine addiction, neuroactive ligand-receptor interaction, dopaminergic synapse etc. As we know, addiction shares common features with traditional learning models, for example, long term potentiation at excitatory synapses of the hippocampus, which is a well-known molecular model of learning and memory. In the present study, we will investigate the effects of IMPX977 on long term potentiation (LTP), one of the classical characteristic of RTT is severe mental retardation, learning and cognitive disabilities (Banerjee et al., 2019; Fabio et al., 2016; Hao et al., 2015). If IMPX977 is able to affect LTP, that will be a great support for IMPX977 to as a potential new drug for RTT treatment.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice [(20 ± 2) g] were provided by the Experimental Animal Center of the Chinese Academy of Medical Sciences, Beijing, China. Mice were housed in a temperature and light-control room [(23 ± 1) °C, 12 h light cycle] and had free access to food and water. All animals were handled in accordance with the standards established in the Guide for the Care and Use of Laboratory Animals published by Chinese Animal Use and Care Committees and executed according to the National Animal Law.

2.2. Drugs and reagents

IMPX977 was purchased from Cixi Biological Technology Co., Ltd., Mecp2 antibody was kindly provided by Dr. Hu of Institute of Medicinal Plant Development. Olive oil was purchased from Star Fine Foods Borges USA, Inc. All other chemical reagents in this study were analytical grade.

2.3. Experimental groups

A total of 32 male C57BL/6 mice were obtained from the institute of Laboratory Animal Science, CAMS & PUMC (Beijing, China), mice were caged in an air-conditioned, light-controlled animal room, and were fed mice chew and water adlibitum. Mice were randomly divided into four groups: control (distilled water), vehicle (Olive oil), IMPX977 (5 mg/kg and 15 mg/kg), mice were treated with P.O, and IMPX977, olive oil or distilled water every other day morning. Three mice from each group were anesthetized with chloral hydrate and then sacrificed after two weeks administration. The other mice in each group were arranged for the electrophysiological recording.

2.4. Electrophysiological recording

2.4.1. Tissue preparation

The methods for preparing and maintaining the hippocampal slices have been reported previously. The rats were deeply anesthetized with diethyl ether and decapitated. The brain was rapidly removed and immersed for 2 min in ice-cold artificial cerebrospinal fluid (ACSF: 124 mmol/L NaCl, 3.0 mmol/L KCl, 24 mmol/L $NaHCO_3$, 1.2 mmol/L kH_2PO4, 1.3 mmol/L $MgSO_4$, 10 mmol/L *D*-glucose and 2.5 mmol/L $CaCl_2$), which was continuously bubbled with carboge (95% O_2 + 5% CO_2) at room temperature for at least 1 h before recording, and had a final pH of 7.3. The brain block which contained hippocampus was mounted on the stage of a vibratome (VT1200, Leica Microsystems, Germany). The brain block was cut with a vibratome into 350 μm-thick transverse slices in ice-cold ACSF. The parameters of the vibratome were set program (speed: 0.16 mm/s, amplitude: 0.90 mm). The slices were then transferred to a recording interface chamber and perfused with carbogenated ACSF at (30 ± 1) °C for at least 1 h before the LTP experiments started.

2.4.2. Long term potentiation induction in vitro

After the slices incubated for 1 h, they were transferred to a recordingchamber and perfused continuously with Artificial Cerebrospinal Fluid (ACSF) at the speed of 2 mL/min at room temperature. Recordings electrode was made of borosilicate glass capillaries (2–3 MΩ) filled with 4 mol/L NaCl. As shown in Fig. 1, the field excitatory postsynaptic potential (fEPSP) was employed as a measure of excitatory synaptic transmission in the CA1 region. Under visual control, a stainless steel concentric bipolar electrode was inserted into the Schaffer collaterals fibers and the recording electrode was inserted into CA1 region close to the granular layer. Traces were filtered at 3 kHz, digitized at 10 kHz, and stored on a personal computer. Recordings were performed using an amplifier (Model 1800, A.M. System Instruments, USA). fEPSP were evoked by test stimuli (0.033 Hz, 0.1 ms duration) via two bipolar tungsten electrodes insulated to the tip. Control test pulses were given every 30 s. Pulse intensity was set for generating 1/3–1/2 of fEPSP induced by maximum intensity. LTP was induced by HFS (High frequency stimulation) protocol (100 Hz, 100 pulses). Baseline stimuli were delivered more than 30 min after fEPSP reaching to the steady state. Recordings were continued for 50 min after HFS. The magnitude of LTP was calculated as a percentage of the averaged fEPSP slope value after HFS achieving the baseline fEPSP slope value. Signals were amplified with a differential amplifier (Digidata 1440A, Axon, USA), recorded using AxoScope acquisition software and analyzed with the Clampfit 10.2 software package.

2.4.3. Statistical analysis

All data were shown as the mean ± S.D. All graphics were drawn with Origin 7.5 software or Gel-Pro analyzer 4.0. Statistical com-

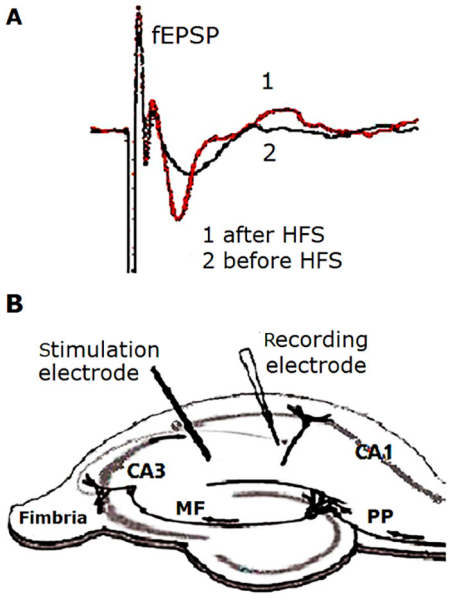

Fig. 1. Cross section structure diagram of mice hippocampal slices. (A) fEPSP was recorded and calculated according to different manipulation after HFS, long term potentiation was induced. (B) stimulation electrode was put on the Schaffer collaterals and recorded the postsynaptic field potential responses in the apical dendritic region of area CA1.

parisons among different groups were analyzed using the Student's *t*-test. $P < 0.05$ was regarded as statistically significant.

2.5. Western blotting analysis

Preliminary experiments were conducted to determine the optimal amount of protein to load on the gel for each antibody. Mecp2 (Mecp2 antibody was kindly offered by Dr. Hu (Hu et al., 2006). Protein levels were assayed in crude homogenates. Tissues were homogenized by sonication in ice-cold radioimmunoprecipitation assay buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, and 1 mmol/L sodium orthovanadate in phosphate-buffered saline buffer, pH 7.4). Protein concentrations were determined using Pierce BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL), and homogenates were diluted to a concentration of 2 mg/mL with 2xSDS-polyacrylamide gel electrophoresis loading buffer. Samples were boiled for 6 min. Sample protein was separated on 8% polyacrylamide minigels. Proteins separated by electrophoresis were transferred to Immobilon-polyvinylidene difluoride membranes (Millipore Corporation) using western blot transfer apparatus. Nonspecific binding to membranes was prevented by blocking for 60 min at room temperature in TBS solution containing 5% nonfat dry milk. Membranes were then probed with Mecp2 antibody. Membranes were rinsed three times with TBS and then incubated with 1:5000 dilution of horseradish peroxidase-labeled secondary antibody in TBS solution containing 0.25% nonfat dry milk for 90 min at room temperature. After washing three more times, antibody complex was visualized by chemiluminescence using a kit from Pierce Biotechnology. Changes in the immunoreactivity values were expressed relative to average value (defined as 100 value), resulting in a mean ± S. D. All these procedures used reagents precooled at 4 °C. Cortex, and hippocampus were dissected out into 10 volumes of ice-cold 10 mmol/L Tris/HCl pH 7.4 buffer and homogenized by a polytron. The samples were then processed for Western blot analysis as described above.

2.6. Data analyses and Statistics.

Western blots were quantitated using standard methods. Pooled control and treated samples were run side by side. The density of the Mecp2 band in the treated sample was divided by the density of the Mecp2 band in the control sample and multiplied by 100 to yield a percent of control. This was done in at least three independent experiments. Statistical significance of the Mecp2 Western blotting data was determined by the Student's *t* test.

3. Results

3.1. Effects of IMPX977 on Mecp2 protein expression in cortex and hippocampus in mice.

As shown in Fig. 2, Mecp2 expression of cortex and hippocampus samples from IMPX977 (15 mg/kg), vehicle and control group was determined by western blotting, the blots were quantified by NIH IMAGE, Mecp2 protein expression in cortex of IMPX977 administration group was increased by (42 ± 5.5)% and (38 ± 4.3)% in hippocampus individually, and the statistically results showed that there was significant difference between IMPX977 (15 mg/kg) and vehicle group (Student' *t*-test, $^{**}P < 0.01$). No difference between vehicle and control group, olive oil did not affect the Mecp2 expression.

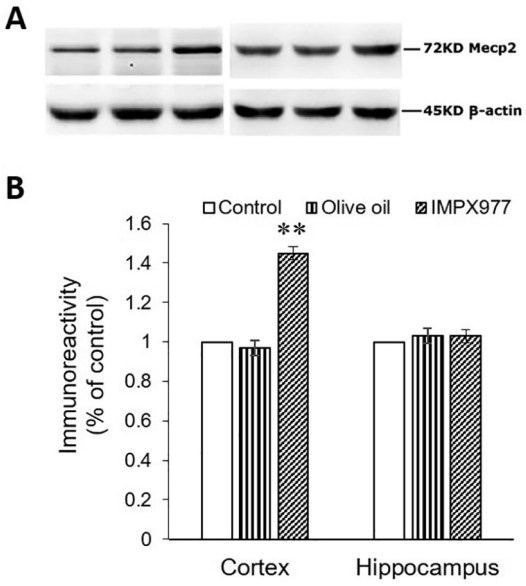

Fig. 2. Western blotting analysis of Mecp2 immunoreactivity levels in mice Cortex and hippocampus in IMPX977 administration ($n = 4$) and control groups ($n = 4$).

(A) Representative blots of Mecp2 and β-actin with the same protein loading in each group; (B) Blots were digitized, and quantified using densitometric analysis (NIH Image software). Changes in immunoreactivity values were expressed relative to their corresponding control (defined as 100% value). $^{**}P < 0.01$ *vs* vehicle group

3.2. Effects of IMPX977 on basal synaptic transmission in mice acute slices.

The evoked response was generated in the stratum radiatum by stimulating at low frequency (0.033 Hz) with single biphasic square wave pulses of 0.1 ms duration per half wave, generated by a constant current isolation unit. The fEPSP slope of IMPX977 treated, olive oil and control mice slices were recorded, for each time point measured during the experiments, five records of evoked responses were averaged. The first six time points, recorded at 5-min intervals, were used as baseline, and all points are shown in relation to the average of these six points.

As shown in Fig. 3, the fEPSP slope of IMPX977 treated slices were (104.3 ± 7.2)%, (99.2 ± 7.5)%, and (101.1 ± 9.2)% at 15, 45, and 60 min, and, the fEPSP slope of the Olive oil were (100.5 ± 6. 9)%, (103.2 ± 7.2)%, (98.3 ± 7.1)% and the control mice showed (97.6 ± 6.8)%, (103.1 ± 7.4)% and (100.2 ± 8.1)% at 15, 45, and 60 min individually. Basal synaptic transmission was not altered in Schaffer collateral-CA1 pathway in IMPX977 (5 and 15 mg/kg) treated mice relative to vehicle controls and no significantly change between vehicle and control mice neither.

The evoked field EPSPs was recorded in Schaffer collateral-CA1 LTP hippocampal pathway and quantified by measuring the rising slope of the EPSP. The average amplitude of fEPSP during 30 min of stable baseline responses was defined as 100%. All data were presented as mean ± SD of six observations

3.3. Effects of IMPX977 on synaptic transmission of schaffer collateral-CA1 LTP pathway in acute mice hippocampal slices

fEPSP was recorded 30 min before HFS as the baseline, and fEPSP slope was quantified as percent of the average slope of baseline (Fig. 4). When HFS was given, slope of fEPSP was significantly increased, the amplitude of control slices showed (156.3 ± 13.5)%, (167.3 ± 16.5)%, (168.2 ± 14.3)%, (167.7 ± 14.9)% of baseline at peak,

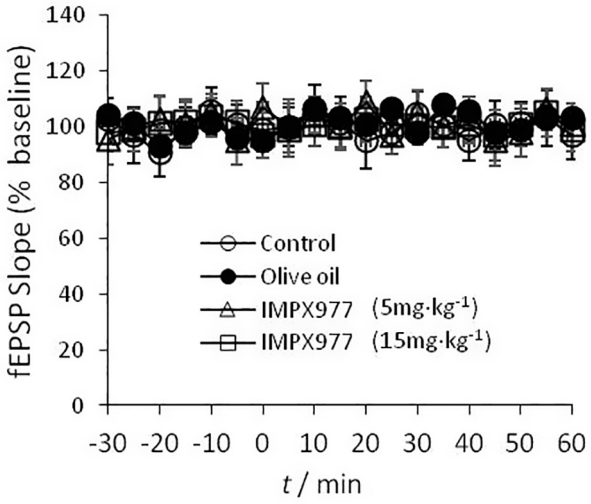

Fig. 3. Basic synaptic transmission in acute slices of IMPX977 administration and control group mice.

10, 30, 40 and 60 min after HFS. In contrast, the relative value of fEPSP slope from vehicle group were (154.0 ± 15.6)%, (147.5 ± 12.4)%, (146.8 ± 15.7)% and (153.2 ± 13.8)% at peak, 10, 30, 40, and 60 min after HFS compared with baseline parameter. IMPX977 (5 mg/kg) enhanced fEPSP slope value significantly, the relative slope were (180.3 ± 13.2)%, (182.7 ± 18.0)%, (175.6 ± 12.8)% and (179.6 ± 11.8)% at peak, 10, 30, 40, and 60 min after HFS (n = 6, Student' t-test, $P < 0.05$). In contrast, IMPX977 (15 mg/kg) increased the fEPSP slope to (191.0 ± 21.4)%, (190.8 ± 19.7)%, (201.4 ± 20.4)% at peak, (191.4 ± 19.4)% at 10, 30, 40, and 60 min after HFS. Compared to the vehicle group, IMPX977 (15 mg/kg) had significant enhanced on the relative value of PS amplitude (n = 6, Student' t-test, $P < 0.01$).

4. Discussion and conclusion

RTT is a progressive neurologic developmental disorder caused by mutations in the MECP2 gene and one of the most common causes of mental retardation, it is transmitted as an X-linked dominant trait, therefore almost exclusively affecting females. It is unclear how *Mecp2* mutations lead to dysfunction of the nervous system in RTT, and no effective treatments for RTT are available (Lp et al., 2018; Liyanage and Rastegar, 2014; van Karnebeek et al., 2016). However, targeting Mecp2 is becoming an important strategy to overcome RTT disorder. IMPX977 was reported to increase Mecp2 protein expression level in female rats, upregulate Mecp2 target genes and increased BDNF protein expression in the previous studies (Hu et al., 2017; Zhao et al., 2019). We also found that the Mecp2 protein expression levels both in cortex and hippocampus were increased in mice after IMPX977 treatment in the present study. IMPX977 mediated upregulation of Mecp2 protein expression, and also affected its downstream target genes (Zhao et al., 2019). As a classic methylated-DNA- binding protein, MeCP2 plays a key role in mediating transcriptional repression which is widely distributed in mammalian somatic cells and present at highest levels in brain, lung and spleen (Shahbazian et al., 2002), moreover, MeCP2 protein expresses in a majority of neuron, it is associated with maturation of the central nervous system and in forming synaptic contacts. View collectively, we speculate IMPX977 may contribute to RTT treatment.

The upregulated Mecp2 protein level could strengthen the synaptic efficacy as well as improve the neural circuit and signal transduction pathway related to MeCP2 (Lo et al., 2016). Accordingly, we investigated the effects of IMPX977 on synaptic transmission of Schaffer-CA1 pathway in acute hippocampal slices, the Schaffer collateral is located between the CA3 and CA1 region in hippocampus. Schaffer collaterals are the axons of pyramidal cells that connect two neurons (CA3 and CA1) and transfer information from CA3 to CA1. LTP is a long-lasting activity-dependent

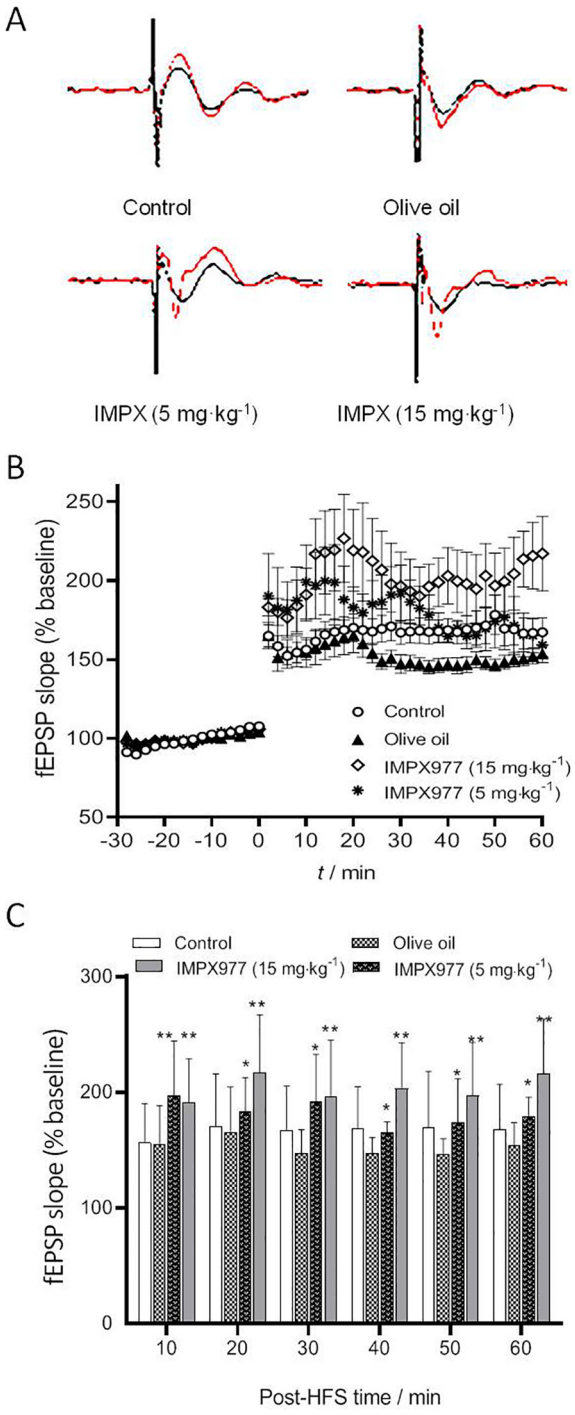

Fig. 4. Effects of IMPX977 (5 mg/kg and 15 mg/kg) on Schaffer collateral-CA1 pathway LTP induced by HFS (100 HZ, 100 us duration) at acute hippocampal slices. (A) Representative traces of EPSP before and after HFS. (B) Time curses of effects of HFS on EPSP. (C) Cumulative histograms of averaged EPSP slopes measured 10, 20, 30, 40, and 50 min post-HFS in five conditions (n = 6). Field EPSP was recorded 30 min before HFS was applicated, the average amplitude of fEPSP slope was defined as 100%. All data were presented as mean ± S.D of six observations.* $P < 0.05$, ** $P < 0.01$ *vs* vehicle group.

enhancement in excitatory synaptic strength following the delivery of a brief, high-frequency train of electrical stimulation, which is generally considered the closest neural model for cellular mechanism involved in learning and memory storage (Cobar et al., 2017; Castellano et al., 2017). It is interesting that, IMPX977 elevated LTP induction in the CA1 region of hippocampus in the present study, which means that IMPX977 may improve the cognition ability because LTP is thought to represent a very likely cellular mechanism underlying some forms of learning and memory. It was well known that one of the key features of Rett syndrome was cognitive disabilities. Therefore, it is possible that IMPX977 accelerate learning and memory ability, however, this certainly need further observations. Particularly, Rett animal models which develop progressive neurological defects should be used to testify the hypothesis (Kyle et al., 2018; Guy et al., 2007; Kee et al., 2018; Rakela et al., 2018). Researchers found that there was an imbalance between excitatory and inhibitory neural network properties due to Mecp2 gene deletion, one possibility would be that the expression and transport of glutamatergic and GABAergic synaptic components was altered, for example the presence of GABA and glutamate receptors (Calfa et al., 2015; Jacque et al., 2018; Sun et al., 2019). Li et al. reported that LTP is occluded in Mecp2 KO mice by already potentiated synapses, the higher surface levels of GluA1-containing receptors are consistent with altered expression levels of proteins involved in AMPA receptor trafficking (Li et al., 2016). We hypothesize that IMPX977 may affect glutamatergic synaptic components which was contribute to LTP alteration in Schaffer-CA1 pathway.

Besides, LTP at the Schaffer collateral to CA1 synapse is NMDA receptor-dependent and expressed in part via postsynaptic mechanisms, for example, the elevated calcium, a variety of calcium-activated enzymes, the membrane depolarization and the components of the synaptic infrastructure that control its function etc., also many components of the postsynaptic density serve as targets of the MDA receptor in terms of its triggering downstream molecular consequences. View collectively, these are possible functional categories to help organize the complex biochemical machinery of NMDA receptor regulation which is tightly related to Schaffer-CA1 LTP as IMPX977 showed in the present study.

These results suggested that IMPX977 supplement may contribute to relieve RTT symptoms, the detailed mechanism, however, needs to be further determined.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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