Decreased Glial GABA and Tonic Inhibition in Cerebellum of Mouse Model for Attention-Deficit/Hyperactivity Disorder (ADHD)

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About 5~12% of school-aged children suffer from the Attention-Deficit/Hyperactivity Disorder (ADHD). However, the core mechanism of ADHD remains unclear. G protein-coupled receptor kinase-interacting protein-1 (GIT1) has recently been reported to be associated with ADHD in human and the genetic deletion of GIT1 result in ADHD-like behaviors in mice. Mice lacking GIT1 shows a shift in neuronal excitation/inhibition (E/I) balance. However, the precise mechanism for E/I imbalance and the role of neuron-glia interaction in GIT1 knockout (KO) mice have not been studied. Especially, a possible contribution of glial GABA and tonic inhibition mediated by astrocytic GABA release in the mouse model for ADHD remains unexplored. Therefore, we investigated the changes in the amount of GABA and degree of tonic inhibition in GIT1 KO mice. We observed a decreased glial GABA intensity in GIT1 KO mice compared to wild type (WT) mice and an attenuation of tonic current from cerebellar granule cells in GIT1 KO mice. Our study identifies the previously unknown mechanism of reduced astrocytic GABA and tonic inhibition in GIT1 lacking mice as a potential cause of hyperactivity disorder.

Key words: ADHD, GIT1, tonic inhibition, glia, astrocyte, GABA

INTRODUCTION

ADHD is a well-known neurodevelopmental disorder with traits of inattention, hyperactivity, and impulsivity [1]. Current statistics has shows that number of patients suffering from ADHD increased from 7% in 2003 to almost 10% in 2012 [2, 3]. Due to this prevalence of ADHD, many researchers have carried out numerous studies and found that various genetic and environmental factors are important to manifest ADHD in the developing brain [4]. However, the core mechanism of ADHD is still not known [5]. For example, there has been a long debate on how hyperactivity and attention deficits, the main traits of ADHD, are associated with motor control and cognitive function [6]. Most of the previous studies have been focused on the cerebral circuits, which include prefrontal cortex and basal ganglia [7, 8]. However, some of recent studies have suggested that cerebellum is associated not only to motor control but also to cognitive functions [9, 10]. Consistently, a recent study on cerebellar contribution in ADHD reported that...
there is a decreased volume of cerebellum during the development [11]. Furthermore, some recent studies have revealed that tonic inhibition in cerebellum can modulate motor function [12] and that tonic GABA is released from cerebellar glial cells [13]. These findings led us to question about the relationship between ADHD and tonic GABA release in cerebellum.

To delineate the mechanisms underlying ADHD investigators have been using various animal models [14]. These models include spontaneously hypertensive rat (SHR), dopamine transporter knockout mice (DAT1 KO), coloboma mouse mutant (Cm), and G-protein coupled receptor kinase 1 knockout mice (GIT1 KO) [16]. All of these models show attention deficit, impulsivity and hyperactivity [14]. However, these models still require more detailed investigations for better understanding of the etiology [16]. One of the mostly used animal model, SHR shows all of the major ADHD traits, except for that there is no sex difference, which appears in human [3, 14, 17]. Even though DAT1 KO mice show hyperactivity and impulsivity, DAT1-knockin mice carrying the cocaine-insensitive mutation exhibit reduced DAT activity [18]. Moreover, ADHD patients do not show a reduction of dopamine transporter [16]. The Cm mice are only viable in heterozygote (Cm+/−) [18]. This model carries a mutation in SNAP-25 gene, and a human genetic study to find a relationship between SNAP-25 and ADHD is needed [16, 18].

In this study, we used GIT1 KO mice. This model shows prominent traits of ADHD, such as hyperactivity and impaired learning and memory [19]. The hyperactivity in GIT1 KO mice is reversed by amphetamine and methylenidate, which are the commonly used psychostimulants to treat ADHD [19]. Also, GIT1 KO mice show an inhibitory synapse transmission [19], this can be the reason for ADHD in human [20]. Therefore, we investigated the possible role of neuron-glia interaction in terms of tonic GABA release from astrocytes in cerebellum from GIT1 KO mice [9], which in the previous studies showed impaired motor coordination [20, 21] and astrocytosis in basal ganglia pathway [22].

MATERIALS AND METHODS

Animals

The male of littermate mice which are GIT1 wild type and knockout type in the age of 6 weeks were used. The wild type and GIT1 KO mice were in hybrid strain of 129S1/SvImJ and C57BL/6. All experimental procedures described below were performed in accordance with KIST (Seoul, Korea) and Dankook University Animal Experimentation Guidelines (approval number DKU-17-022, Cheonan, Korea).

Primary cortical astrocyte cultures

Cerebellar cortices were dissected from P0–P2 postnatal GIT1 KO or WT mice, cleared of adherent meninges, minced, and dissociated into a single-cell suspension by trituration. Dissociated cells were plated onto 12-mm glass coverslips coated with 0.1 mg/ml poly D-lysine. Cells were grown in DMEM supplemented with 25 mM glucose, 10% heat-inactivated horse serum, 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 1000 units/ml penicillin-streptomycin. Cultures were maintained at 37°C in humidified atmosphere of 5% CO₂.

HPLC analysis

Amino acid content was derivatized with o-phthalaldialdehyde (OPA) and quantified with UV Diode Array Detection (DAD). OPA-derivatized samples were collected with a programmed autosampler and injected onto a Zorbax Eclipse Plus C18 column with detection at 338 nm (reference, 390 nm). Mobile phase A was 40 mM Na₂HPO₄ (pH 7.8) and phase B was acetonitrile–methanol–water (45:45:10, v:v:v). The flow rate was 2 ml/min with a gradient condition that allowed for 1.9 min at 0% B and a rise to 26% B over a 12.5-min step. Subsequent washing at 100% B and equilibration at 0% B was performed within a total retention time of 15 min. Reagents for OPA derivatization and all equipment for HPLC analysis were obtained from Agilent Technologies.

Immunohistochemistry

Mice aged P42–P49 were deeply anesthetized by 2% avertin (20 µg/g) and perfused with 0.1M PBS (Phosphate buffered saline) followed by ice cold 4% PFA (paraformaldehyde). Excised brains were post-fixed overnight in 4% PFA at 4°C and immersed in 30% sucrose for 48 hrs for cryo-protection. Paraffgittal cerebellar sections (30 µm), rinsed in PBS three times and incubated 1 hr at RT with blocking solution (0.3% Triton-X, 2% normal serum in 0.1 M PBS). Sections were incubated overnight in a mixture of the following primary antibodies with blocking solution at 4°C on shaker; chicken anti GFAP antibody (1:500; Millipore) and guinea-pig anti GABA (1:200; Sigma). After washing three times in PBS, sections were incubated with corresponding secondary antibodies; conjugated Alexa 647 goat anti guinea-pig antibody (1:200; Jackson ImmunoResearch Inc.) and Alexa 488 donkey anti chicken antibody (1:200; Jackson ImmunoResearch Inc.), for two and a half hours, followed by one rinse in PBS, and incubated once with DAPI (1:1000) in PBS. After incubated with DAPI, followed by one rinse in PBS. Then mounted with an anti-fade mounting medium. A series of fluorescence images was obtained with confocal microscope (Zeiss, LSM 700) and images were processed for later analysis using ImageJ program and ZEN 2010 imaging software.
Slice recording

Adult mice aged P42-P49 were deeply anesthetized with halothane. After decapitation, the brain was quickly excised from the skull and submerged in ice-cold cutting solution that contained (in mM): 126 NaCl, 24 NaHCO3, 1NaH2PO4, 2.5KCl, 2.5CaCl2, 2MgCl2, and 10D(+)-glucose, pH 7.4. Whole solution was gassed with 95% O2 and 5% CO2. After trimming the cerebellar brain, 250 μm parasagittal slices were cut using a vibratome (DSK LinearSlice, Kyoto, Japan) with a blade (DORKO, Seoul, Korea) and transferred to extracellular ACSF solution (in mM): 126 NaCl, 24 NaHCO3, 1NaH2PO4, 2.5KCl, 2.5CaCl2, 2MgCl2, and 10D(+)-glucose, pH 7.4. Slices were incubated at room temperature for at least one hour prior to recording. Slices were transferred to a recording chamber that was continuously perfused with aCSF solution (flow rate=2 ml/min). The slice chamber was mounted on the stage of an upright Olympus microscope and viewed with a 60X water immersion objective (NA=0.90) with infrared differential interference contrast optics. Cellular morphology was visualized by CCD camera and Axon Imaging Workbench software. Whole-cell recordings were made from cerebellar granule cell somata located in lobules 2~5. The holding potential was -60 mV. Pipette resistance was typically 5~8 MΩ for granule cells and the pipette was filled with an internal solution (in mM): 135 CsCl, 4 NaCl, 0.5 CaCl2, 10 HEPES, 5 EGTA, 2 Mg-ATP, 0.3 Na2-GTP, 10 QX-314, pH adjusted to 7.2 with CsOH (278~285 mOsmol). Electrical signals were digitized and sampled at 50 μs intervals with Digidata 1440A and Multiclamp 700B amplifier (Molecular Devices) using pCLAMP 10.2 software. Data were filtered at 2 kHz.

Data analysis and statistical analysis

Off-line analysis was carried out using Clampfit, Minianalysis, SigmaPlot and Excel software. The significance of data for comparison was assessed by Student’s two-tailed unpaired t-test. In general, data distribution was assumed to be normal but this was not formally tested. The data distribution was assumed to be normal. Data are presented as mean±SEM (standard error of the mean). Levels of statistical significance are indicated as follows: *(p<0.05),**(p<0.01),***'(p<0.001).

RESULTS

To determine the change of gliotransmitters in ADHD, we directly measured the amount of released extracellular glutamate and GABA from the cerebellar primary glia culture and performed the subsequent analysis of the media and cell lysate by high performance liquid chromatography (HPLC). We found that extracellular GABA concentration showed a slight decreasing tendency but not statistically different in GIT1 KO compared to WT (Fig. 1, middle). In addition, E/I ratio, calculated by dividing glutamate by GABA concentration, was slightly but not significantly increased in GIT1 KO compared to WT (Fig. 1, right). There was no difference in the level of glutamate (Fig. 1, left).

Next, to confirm the decrease of astrocytic GABA contents in cerebellum, we performed immunohistochemistry using the commercially available anti-GABA antibody in the cerebellar cortical tissue. We observed a decreased glial GABA intensity in GIT1 KO compared to WT by using a confocal microscopy (Fig. 2A–D). The intensity of GABA in glial cells (GFAP positive GABA pixel) in cerebellum of GIT1 KO was decreased by about 60% of WT level. There was no significant difference in GFAP intensity between GIT1 KO and WT (Fig. 2E).

It is possible that the decreased GABA content in cerebellar glial cells of GIT1 KO can induce a less tonic GABA release and affect the neuronal activity on the neighboring neurons and E/I ratio.

![Fig. 1](after primary astrocyte culture, major gliotransmitters in media from wild type (WT) or GIT1 Knock Out (GIT1 KO) mice are analyzed by HPLC. Bar graph shows concentration of excitatory transmitter, glutamate (left); inhibitory transmitter, GABA (middle); and excitatory/inhibitory ratio (right). GIT1 KO mice show lower GABA concentration and higher E/I ratios (Glutamate p=0.8, GABA p=0.60, E/I ratio p=0.68, analyzed by unpaired t test, n=22; from 15 mice of WT and 7 mice of KO).}
test this possibility, we measured the tonic inhibition current from granule cells in cerebellar slices by measuring the baseline current shift upon GABA_AR antagonist (50 μM GABAzine). We detected a significant decrease in tonic inhibition current from cerebellar granule cells of GIT1 KO mice compared to WT (Fig. 3A). Tonic inhibition current of GIT1 KO was significantly decreased by 85% of WT level (Fig. 3B, left). There was no significant difference in the full activation current which was induced by 5 μM GABA (Fig. 3A, B, middle). However, the % of full activation, which was calculated by dividing the tonic inhibition current by full activation for each recorded cell, showed a significant reduction in GIT1 KO mouse compared to WT (Fig. 3B, right). The % of full activation is a good indicator of the GABA release component. Therefore, the results indicate that there was a significant decrease in the tonic GABA release from cerebellar glial cells including Bergmann glia in purkinje cell layer and lamellar astrocyte in granule cell layer in GIT1 KO mice, without affecting the GABA_AR level in granule neurons. In contrast, we could not find any significant change in the spontaneous inhibitory post-synaptic current (sIPSC) amplitude and frequency between WT and GIT1 KO (sIPSC amplitude: 16.2±2.2 pA (WT); 14.2±1.1 pA (KO), sIPSC frequency: 0.2±0.04 Hz (WT); 0.2±0.02 Hz (KO)), indicating that the synaptic GABA

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**Fig. 2.** Representative confocal microscope images of immunohistochemistry in cerebellum of WT (A, C) and GIT1 KO (B, D) at 20X (A, B) and 40X (C, D). Arrowhead indicates GFAP-negative cells and black asterisks indicates GFAP-positive cells. Scale bar indicates 50 μm in (A, B) and 20 μm in (C, D). (DAPI : Blue, GFAP : Green, GABA : Red). The GFAP intensity of (A) and (B) were quantified in (E). Both GFAP intensity and GABA intensity in GFAP-negative cells shows no significant difference between WT and GIT1 KO mice. However, GABA intensity in GFAP-positive cells shows significant decrease in the GIT1 KO mice (n=22, from 4 mice of WT and 4 mice of KO, ***p<0.005).
release was not changed in the GIT1 KO mice.

DISCUSSION

As the ADHD is complex disorder affected by both genetic and environmental factors, the availability of appropriate animal models is a big step towards understanding the mechanism of the disorder [14]. Therefore, molecular and cellular studies using animal models for the pathological states will help us to understand related mechanisms in human. In this study, we suggest an involvement of glial GABA in GIT1 KO mice, in that there is a decreased GABA level in astrocytes of the cerebellum, which results in less tonic inhibition current mediated by diminished tonic GABA release, leading to an increase of E/I ratio and hyperactivity (Fig. 4).

In this study, the HPLC experiment from cultured astrocytes had not revealed a significant difference in GABA level between WT and KO ($n=18$ from 4 mice of WT and 5 mice of KO; $^*p<0.05$, $^{**}p<0.01$).

Fig. 3. (A) Representative traces of whole cell patch clamp recordings from granule cells in cerebellar slices in WT (Left) and GIT1 KO (Right). 5 μM of GABA is treated on the slices for the full activation of all GABA receptors. The tonic current was measured as a blocked current by 10 μM GABAzine treatment (black arrow). The measurements are summarized in (B). Tonic current is significantly decreased in KO mice. The full activation current with 5μM of GABA is not different between WT and KO ($n=18$ from 4 mice of WT and 5 mice of KO; $^*p<0.05$, $^{**}p<0.01$).

Fig. 4. Comparable schematic diagram of cerebellar cortex in WT and GIT1 KO mice. Reduced GABA in the astrocytes was observed in GIT1 KO compared to WT (Fig. 2E). This reduced astrocytic GABA induced less tonic GABA less tonic currents mediated by tonic GABA release via Best1 channels. Conclusively, attenuation of tonic inhibition make increase of E/I balance and can explain hyperactivity for ADHD. BG, Bergmann glia; GC, Granule cell; LA, Lamellar astrocyte; PF, Parallel fiber.
and KO (Fig. 1). These results might reflect the environmental difference between cultured cells grown in culture and acute cerebellar slices. Another possibility is that the discrepancy might be due to the age of culture (P0-P2) and acute cerebellar slice (P42-P49). Another reason for no significant change in GABA level between WT and KO could be due to the low sensitivity in GABA from culture media by HPLC. The level of GABA detected from the culture media is expected to be extremely low at the concentration range of picomolar to subnanomolar.

We have demonstrated that the cerebellar tonic GABA current is significantly reduced in GIT1 KO compared to WT (Fig. 3). Tonic inhibition is shown to be mediated by constant accumulation of GABA, which is released from glial cells, in the extracellular space [13]. Because of its sustained nature, tonic inhibition can regulate the excitability of the neurons in the brain [13]. Moreover, altered tonic inhibition in cerebellum can induce motor impairments [12]. Because the cerebellum is known to control the motor function [7, 23], the increased E/I ratio in GIT1 KO reflects the decreased tonic GABA and causes hyperactivity in ADHD.

We have provided new lines of evidence that there is a mechanism for neuron-glia interaction which controls E/I ratio in ADHD model mouse. Such mechanism can be useful for studying the role of astrocytic GABA in physiological conditions as well as pathophysiological conditions. These conditions include various psychiatric disorders such as depression, seizure and autism, in which the E/I balance has been compromised. In this study, we have focused on the involvement of cerebellar glial cells in the hyperactivity. These new ideas enlighten us to better understand the etiology of ADHD possibly in terms of involvement of glial cells and neurons and neural circuits of the cerebellum [5, 24]. In light of the recent observation showing the astrogliosis in basal ganglia pathway in GIT1 KO mouse [22], it would be interesting to see if there is also tonic inhibition current increase and astrocytic GABA increase in basal ganglia. Many ADHD studies in the past have focused on the neural circuits including frontostriatal circuits; this circuits include prefrontal cortex to basal ganglia. Based on the current study, cerebellar circuits should be included in ADHD studies. Both cerebellum and basal ganglia might play an important role in motor function in ADHD [10]. Future investigations will allow us to determine how cerebellar dysfunction and/or basal ganglia is involved in ADHD.

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