GIT1, a multifunctional signaling adaptor protein, is implicated in the development of dendritic spines and neuronal synapses. GIT1 forms a signaling complex with PIX, RAC, and PAK proteins that is known to play important roles in brain development. Here we found that Git1-knockout (Git1\(^{-/-}\)) mice show a microcephaly-like small brain phenotype, which appears to be caused by reduced neuronal size rather than number. Git1\(^{-/-}\) mice also show decreased dendritic spine number without morphological alterations in the hippocampus. Behaviorally, Git1\(^{-/-}\) mice show impaired motor coordination and learning and memory. In addition, adult dGit Drosophila mutants show decreased brain size and abnormal morphology of the mushroom body. These results suggest that GIT1 is important for brain development in both rodents and flies.

**Key words:** GIT1, dGit, Microcephaly, Brain Development

GIT1 and GluN3A impairs synaptic localization of the Git1-PIX signaling complex and thus downregulates RAC1 activation and spine formation [6]. The Ephrin-B-Eph receptor signaling pathway phosphorylates GIT1 to regulate spine and synapse formation [7]. Synaptic role of GIT1 has been further supported through *in vivo* studies as Git1\(^{-/-}\) mice show impaired spine development [8]. These studies collectively suggest the essential role of GIT1 in spine and synapse formation.

GIT1 is also expressed at inhibitory synapses and interneurons and plays an important role in inhibitory transmission. GIT1-βPIX-RAC1-PAK3 signaling pathway is important for the migration of interneurons [9]. Depletion of GIT1 expression results in decreased Rac1 and PAK3 activation and reduced levels of parvalbumin expression [10]. Smith et al. identified the interaction between GIT1 and GABA\(_\lambda\) receptor, which promotes surface expression of GABA\(_\lambda\) receptor through the βPIX-RAC1-PAK signaling pathway [11]. Taken together, GIT1 regulates both excitatory and inhibitory synaptic transmission, which is critical for excitatory and inhibitory balance.
The importance of the GIT1-βPIX-RAC1-PAK3 signaling complex in brain development has been supported by a series of studies. A Mutation of PAK3 is associated with X-linked mental retardation associated with various behavioral and anatomical manifestations including impaired motor coordination, hyperactivity and microcephaly [12, 13]. Mutant mice with a double knockout of Pak1 and Pak3 also display reduced brain size and hyperactivity [14]. Chromosome 13q34 deletion which removes ARHGEF7/PIX is associated with microcephaly [15], and microcephaly-like reduction of brain size has been identified in Rac1-null mice [16]. GIT1 is also important for the development of other tissues, such as bone development [17, 18], angiogenesis [19, 20], and muscle development [21].

dGit, a Drosophila ortholog of Git1, shows remarkable conservation with mammalian Git1 both structurally and functionally. dGit forms signaling complex with dPix and dPak, and the dGit-dPix-dPak signaling pathway plays an essential role for the development in Drosophila. For instance, dGit mutants exhibit impaired muscle development [21]. dPix regulates postsynaptic structure and protein localization mainly through dPak [22]. Rac mutations in Drosophila cause defective axon guidance and projection [23]. In addition, dPak regulates synapse formation [24]. These results collectively suggest the conserved role of the Git1-PIX-RAC-PAK signaling complex in invertebrates.

To better understand the functional implication of GIT1 in the development of the brain, we performed immunohistochemical and behavioral analyses to reveal the importance of GIT1 in the brain development. In addition, using adult dGitex21C Drosophila mutants, we investigated the conserved role of Git1 in brain development.

MATERIALS AND METHODS

Generation of Git1−/− mice and dGitex21C Drosophila mutant

Git1−/− mice has been previously described [10]. dGitex21C Drosophila mutant is a generous gift from Dr. Yang and detailed information regarding the dGitex21C mutant is provided in the previous report [21]. Experiments were done in accordance with the guidelines of the Animal Welfare Committee of KAIST, Korea.

Immunohistochemistry

For NeuN (Millipore) staining, brains were isolated from adult mice (2–3 months old) after cardiac perfusion (4% paraformaldehyde). After the post-fixation for 12 h, 50 μm brain sections were obtained by vibratome (Leica Biosystems). Brain sections were washed 3 times with PBS for 10 min, permeabilized with 0.5% TritonX-100 for 30 min, blocked with 5% bovine albumin (BSA) for 1 h, stained with primary antibodies at 4°C for 12 h, stained with secondary antibodies for 1 h, and mounted with Vectashield.

For staining with anti-FASII (Hybridoma Bank), adult brains of dGitex21C mutants were isolated from the head capsules and fixed in 4% paraformaldehyde in PBS containing 0.5% Triton X-100 for 2 h, and washed in PBS containing 0.5% Triton X-100 three times each for 45 min. Brains were incubated with anti-FASII, and then incubated with the appropriate secondary antibody (Jackson Laboratories). For quantitative analysis, images of brain sections were captured with a confocal microscope (x63 objective; Leica Microsystems) and analyzed using ImageJ software (NIH; http://rsbweb.nih.gov/ij/).

DiOlistic spine labeling and image analysis

Brain slices from the transcardially perfused adult mice (2–3 months old) were labeled by the ballistic delivery of the lipophilic dye DiI as previously described [25]. Dendrites in the proximal stratum radiatum region of the hippocampus were analyzed to quantify spine density, width, and length. For quantification, MetaMorph software (Molecular Devices) was used.

Catwalk analysis

Gait pattern of wild-type and Git1−/− mice was analyzed using Catwalk system (Noldus), as previously described [26]. Detailed information on step patterns was covered in the previous report [27]. Briefly, regular step patterns are categorized into three types, including Cruciate (Ca, Cb), Alternative (Aa, Ab), and Rotate (Ra, Rb), based on the step sequence of frontal and hind paws. Regularity index is the number of regular step patterns relative to the total number of paw placements. Base of support (BOS) is the average width between the frontal paws or the hind paws. Print position is the average distance between the position of the hind paw and the position of the previously placed front paw. Stride length, the distance between consecutive placements of the same paw, and Duty cycle, a percentage of the time for Stand (the duration of paw placement) in a single step, were also measured by the Catwalk system.

Rotarod assay

Motor coordination and motor learning was assessed with a rotarod apparatus. During 4 min session, rotating velocity of the rod was accelerated from 4 rpm to 40 rpm. Experiments were performed during four consecutive days, with two trials per day. Motor performance of the subject mice was measured by the final speed when mice fell off from the rod.
Statistics

Student’s t test was done using Microsoft Excel 2007 (Microsoft). Result of rotarod assay was analyzed by repeated ANOVA using R (http://www.r-project.org/).

RESULTS

Git1–/– mice display microcephaly-like brain size decrement and reduced dendritic spine density

In the previous study, Git1–/– mice showed decreased brain and body size [10]. As the major phenotype of microcephaly is decreased brain to body ratio [28], we examined the ratio to investigate whether the decrement of brain size is the result of general developmental impairment or microcephaly-like brain size reduction. Git1–/– mice showed decreased brain to body weight ratio (Fig. 1A and C), which suggests the decrement of brain size is microcephaly-like phenotype rather than general developmental deficits. As the other Git1–/– mice showed decreased spine density [8], and the GIT1-βPIX-RAC1-PAK3 signaling complex is important for spine morphogenesis [4], we checked dendritic spine morphology in our Git1–/– mice. Spine density (Fig. 1B and D) is significantly decreased in the hippocampus, which is consistent with previous results. However, spine length and width were not altered (Fig. 1B, E and F) in these knockout mice.

Unaltered gross morphology and neuronal density, but significantly decreased neuronal size in Git1–/– mice

To further investigate the impaired brain development of Git1–/– mice, we performed immunohistochemical analysis, which revealed the gross morphology of Git1–/– mice was not different from wild-type littermates except smaller size of the brain (Fig. 2A). The density of neurons in various brain regions was also not

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Fig. 1. Microcephaly-like brain size decrement and reduced dendritic spine density shown in the Git1 knockout brain. (A) Git1–/– mice show reduced brain size compared to wild-type littermates. n=3 (WT), 3 (KO). Scale bar, 1 cm. (B) Representative DiI images of the pyramidal neurons in the CA1 region of the hippocampus. Scale bar, 10 μm. (C) Quantification of the brain to body weight ratio. Git1–/– mice show decreased brain to body weight ratios compared to controls. (D–F) Quantification of the results from (B). Git1–/– mice show decreased dendritic spine density, while spine width and length are unaltered. n=8 (WT), 9 (KO). **p<0.01; Student’s t test. Error bars indicate means±s.e.m.
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Fig. 2. Normal gross morphology and neuronal density, but reduced size of neuronal cell body in the Git1 knockout mouse brain. (A) Representative brain images of various brain regions of wild-type and Git1−/− mice. Scale bar, 1 mm (B) NeuN signal in the cortex (Ctx), CA1 region of the hippocampus (Hip), and ventrobasal thalamus (VB). Scale bar, 20 μm (C) Neuronal density was unaltered in the Git1−/− cortex, hippocampus and ventrobasal thalamus. n=4 (WT), 4 (KO). (D) The size of neuronal cell body was significantly decreased in the Git1−/− mice. n=6 (WT), 6 (KO). **p<0.01, ***p<0.001; Student’s t test. Error bars indicate means±s.e.m.
changed (Fig. 2B). However, the size of neurons was significantly reduced (Fig. 2C), which suggests the decreased brain size shown in \textit{Git1}^{−/−} mice is due to smaller neuronal size rather than decreased number of neurons.

\textbf{\textit{Git1}^{−/−} mice show altered gait and impaired motor coordination}

Microcephaly-affected human patients showed various behavioral phenotypes including mental retardation and hyperactivity [13]. In previous research, \textit{Git1}^{−/−} mice exhibited impaired fear response [29], impaired spatial learning and memory, and ADHD-like hyperactivity [10]. In addition to the impairments in cognitive functions, microcephaly patients also show impaired motor coordination. \textit{Git1}^{−/−} mice displayed normal step patterns in catwalk analysis (Fig. 3A–C) with changes in the print position (Fig. 3D), base of support (Fig. 3E), stride length (Fig. 3F) and duty cycle (Fig. 3G). Along with the altered gait, \textit{Git1}^{−/−} mice showed severe defects in motor coordination and motor learning in rotarod test (Fig. 3H).

\textbf{Adult \textit{dGit}^{ex21C} Drosophila mutants show defects in brain size and mushroom body structure}

To further analyze the role of Git1 in brain development, we observed whether the adult brain development is also impaired in \textit{dGit}^{ex21C} Drosophila mutants. Consistent with \textit{Git1}^{−/−} mice, \textit{dGit}^{ex21C} Drosophila mutants also exhibited severely decreased central brain size (Fig. 4A, B). Moreover, the development of alpha- or/and beta-lobe of the mushroom body, which is known to be important for learning and memory in \textit{Drosophila} [30], was significantly affected by the deletion of \textit{dGit}. (Fig. 4C). However, the penetrance of this impaired mushroom body development was incomplete (Fig. 4D). Among \textit{dGit}^{ex21C} mutant phenotypes with abnormally developed mushroom bodies, the most common pattern of abnormality was early termination of one alpha-lobe (58%; Fig. 4E). Early termination of one beta-lobe (26%) and two alpha-lobes (11%) was less frequent, and divided alpha-lobe was sparse (5%). These results propose the conserved role of the GIT1-\βPIX-RAC1-PAK3 signaling complex in brain development.

\begin{figure}
\centering
\includegraphics{fig3.png}
\caption{\textit{Git1}^{−/−} mice show altered gait and impaired motor learning and coordination. (A) Representative images of gait pattern of wild-type littermates and \textit{Git1}^{−/−} mice in the catwalk analysis. n=8 (WT), 10 (KO). (B) Step patterns of \textit{Git1}^{−/−} mice are not different from those of wild-type littermates. Detailed description of each pattern is provided in the materials and method section. (C) \textit{Git1}^{−/−} mice show high percentage of regularity index patterns comparable to those of wild-type littermates. (D) Print position of right and left paws is significantly altered in \textit{Git1}^{−/−} mice. \textit{Git1}^{−/−} mice place hind paws up to the almost same previous position of their front paws, while wild-type littermate place hind paws on the slightly different position. (E) Base of support of both front and hind paws is significantly decreased in \textit{Git1}^{−/−} mice. (F) Stride length, the distance between consecutive placements of the same paw of \textit{Git1}^{−/−} mice is significantly increased. RF, right front paw; LF, left front paw; RH, right hind paw; LH, left hind paw. (G) \textit{Git1}^{−/−} mice step on the ground more briefly with their paws than wild-type littermates, as shown in decreased duty cycle. (H) Impaired motor coordination and learning of \textit{Git1}^{−/−} mice in the accelerating rotarod test. n=8 (WT), 9 (KO). Repeated Measurements of ANOVA; Repeated Measurements of ANOVA; Main effect of genotype p<0.001, F(1,48)=117.185; Main effect of time p=0.050, F(3,48)=2.753; Main effect of time x genotype p=0.187, F(3,48)=1.653 *p<0.05, **p<0.01, ***p<0.001; Student’s t test and repeated-measures ANOVA. Error bars indicate means±s.e.m.}
\end{figure}
Here, we report a microcephaly-like brain size reduction, decreased neuronal cell body size, and behavioral deficits such as impaired motor coordination and learning in Git1−/− mice. These developmental anomalies were also apparent in Drosophila with

**DISCUSSION**

Fig. 4. Decreased brain size and defective mushroom body structure in adult dGitex21c Drosophila mutants. (A) Left and right images show the brain of control and dGitex21c mutant, respectively. Mutation of dGit causes reduced brain size, as shown by FasII staining. The size of brain is measured by the number of pixels surrounded by the dotted line. Optic lobes were not considered as the central brain area. Scale bar, 100 μm. (B) Quantification of the relative size of the central brain. n=25 (Ctrl), 28 (dGitex21c) (C) Representative images of normal and abnormal structures of the mushroom body in male dGitex21c mutants. Scale bar, 63 μm. (D) 34% of male dGitex21c Drosophila mutants show normal structure of the mushroom body (10 out of 29), while 66% show abnormal structures. (E) Various abnormal structures of mushroom body are shown in dGitex21c mutants. Most of the dGitex21c mutants show terminated alpha or beta lobes, and one dGitex21c mutant shows divided alpha lobes. **p<0.01; Student’s t-test. Data represent mean±s.e.m.**
**dGit** deletion mutation.

Microcephaly, a neurodevelopmental disorder, is characterized by the decreased brain to body ratio, which is often associated with mental retardation and epilepsy [31]. Woods suggested two types of microcephaly; primary microcephaly caused by reduced number of neurons and secondary microcephaly mainly associated with reduced dendritic processes [28]. In **Git1**–/– mice, the number of neurons in various brain regions was unaltered but the size of neuronal cell body was significantly decreased. In addition, other group has reported that **Git1**–/– mice show reduced length of dendrites in hippocampus [8]. Present results and previous reports conjoinly suggest that Git1 might be associated with secondary microcephaly rather than primary microcephaly.

**Git1**–/– mice showed impaired cognitive functions including spatial learning and object recognition deficits [10]. We also found that motor coordination and motor learning are severely impaired in **Git1**–/– mice. In the rotarod test, **Git1**–/– mice displayed poor motor coordination in the first training day compared to their wild-type littermates, and the performance was not improved by subsequent training. In the catwalk analysis, gait patterns were not distinguishable between wild-type control and **Git1**–/– mice. Similar and high percentage of regularity index shown in both wild-type and **Git1**–/– mice suggests both genotypes have normal gait pattern. However, the increased stride length and decreased duty cycle of **Git1**–/– mice might be associated with the hyperactivity shown in **Git1**–/– mice. **Git1**–/– mice moved further in one step cycle due to the increased stride length and showed rapid step cycle which cause faster movement than wild-type control. As the impaired motor coordination and hyperactivity are the comorbidity shown in microcephaly-affected individuals [13, 15], cognitive dysfunctions and hyperactivity shown in **Git1** knockout might be associated with the impaired brain development.

In *D. melanogaster*, it has been reported that the dPix-Rac-dPak signaling complex is important for the normal neural development [22-24]. dGit is important for the exo- and endocytic cycling of synaptic vesicles and muscle development [21, 32]. In the present work, we found reduced size of the central brain and abnormal development of the mushroom body in adult d**Git1**–/– mutants. Together with previous reports, we suggest that subcellular localization and activation of the dPix-Rac-dPak signaling complex is modulated by dGit, and dysregulation of this signaling complex causes severe defects, leading to brain size reduction and abnormal mushroom body development. These results imply the important role of Git in brain development, which is evolutionary conserved in both invertebrates and vertebrates.

In previous works performed by two independent groups using **Git1** knockout by targeting exon2-7 reported normal brain size and morphology [8, 29]. This observation is strikingly different from present study, which might be due to the difference in the strategy to generate **Git1**–/– mice. In this work, we generated **Git1**–/– mice by using gene trapping technique, which ablated the expression of **Git1** completely. The exact cause of this discrepancy shown in two **Git1** knockout models is unclear, but multiple lines of evidence regarding the role of the βPIX-RAC1-PAK3 signaling complex in brain development collectively suggest that the signaling complex is critical for the normal brain development. Individuals with plat53 mutation [13] and chromosome 13q34 deletions that remove ARHGEF7/βPIX gene [15] as well as Rac1-null mice [16] showed microcephaly or microcephaly-like reduction in brain size. In addition, Pak1/3 double knockout also displayed severe brain size reduction possibly caused by the decreased size of neuronal and glial cells [14], consistent with our observations of our **Git1**–/– mice. The products of these mutated/disrupted genes lie downstream of **Git1** in the GIT1-βPIX-RAC1-PAK3 signaling pathway [1], which is strongly suppressed in our mice [10].

In conclusion, severe developmental deficits shown in **Git1**–/– mice as well as adult d**Git1**–/– *Drosophila* mutants indicate the conserved and critical role of **Git1** in brain development. We suggest that **Git1** plays an essential role in brain development by activating and localizing βPIX-RAC-PAK signaling complex.

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