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Abnormal Astrocytosis in the Basal Ganglia Pathway of *Git1*$^{-/-}$ Mice

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Attention deficit/hyperactivity disorder (ADHD) is one of the most common neurodevelopmental disorders, affecting approximately 5% of children. However, the neural mechanisms underlying its development and treatment are yet to be elucidated. In this study, we report that an ADHD mouse model, which harbors a deletion in the *Git1* locus, exhibits severe astrocytosis in the globus pallidus (GP) and thalamic reticular nucleus (TRN), which send modulatory GABAergic inputs to the thalamus. A moderate level of astrocytosis was displayed in other regions of the basal ganglia pathway, including the ventrobasal thalamus and cortex, but not in other brain regions, such as the caudate putamen, basolateral amygdala, and hippocampal CA1. This basal ganglia circuit-selective astrocytosis was detected in both in adult (2-3 months old) and juvenile (4 weeks old) *Git1*$^{-/-}$ mice, suggesting a developmental origin. Astrocytes play an active role in the developing synaptic circuit; therefore, we performed an immunohistochemical analysis of synaptic markers. We detected increased and decreased levels of GABA and parvalbumin (PV), respectively, in the GP. This suggests that astrocytosis may alter synaptic transmission in the basal ganglia. Intriguingly, increased GABA expression colocalized with the astrocyte marker, GFAP, indicative of an astrocytic origin. Collectively, these results suggest that defects in basal ganglia circuitry, leading to impaired inhibitory modulation of the thalamus, are neural correlates for the ADHD-associated behavioral manifestations in *Git1*$^{-/-}$ mice.

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a prevalent psychiatric disorder that affects approximately 5% of children worldwide. It is characterized by inattention, hyperactivity, and impulsivity. Psychostimulants, such as amphetamine and methylphenidate, are frequently used to treat individuals with ADHD. These medications increase the levels of monoamine neurotransmitters at synapses, suggesting that a deficit in monoamines may contribute to ADHD (Biederman, 2005; Swanson et al., 1998; 2007).

In addition, both clinical and genetic studies support the involvement of monoamines in the etiology of ADHD. Several clinical studies have reported dopamine depletion and decreased activity of monoamine-related brain circuits in ADHD (Shaywitz et al., 1977; Volkow et al., 2007). Genome-wide association studies have revealed several chromosomal loci containing dopamine and norepinephrine-related genes that are associated with ADHD (Ogdie et al., 2004). In line with these findings, several ADHD animal models, such as the Spontaneously Hypertensive Rat (SHR), Coloboma mouse with a *Snap-25* mutation, and dopamine transporter null (*Dat*$^{-/-}$) mouse, have been consistently reported to show alterations in the dopaminergic system (Russell, 2002; Sontag et al., 2010). Taken together, these clinical, genetic, and animal studies suggest a dopamine deficit as the most prominent hypothesis for ADHD etiology.

The dopaminergic signaling pathway incorporates interconnected brain regions that form the basal ganglia (cortico-striato-pallido-thalamic) circuit (DeLong and Wichmann, 2007). Dopaminergic neurons in the striatum can regulate thalamic nuclei *via* a direct or indirect pathway that involves the globus pallidus (GP) (Anaya-Martinez et al., 2006). Neuroimaging and neuroanatomical studies have shown that individuals with ADHD display changes in the size and activity of various brain regions, including the frontal cortex, cerebellum, and subcortical structures, such as the caudate nucleus, putamen, GP, and thalamus (Biederman, 2005; Dickstein et al., 2006; Gerring et al., 2000; Ivanov et al., 2010; Qiu et al., 2009; Swanson et al., 1998). These results, together with the observation that some dopaminergic pathways have their nerve terminals in the caudate nucleus and putamen, suggest that defects in the basal ganglia pathway may be neural correlates of ADHD.

In addition to the dopamine deficit hypothesis, there is evidence to suggest a role for astrocytes in ADHD pathophysiology. Astrocytes are important for modulation of synaptic transmission, GABA-mediated tonic inhibition, glutamate metabolism, and supply of nutrients to neurons (Araque et al., 1998; Lee et al., 2010; Sonnewald et al., 1997). Their impairment is related to various neurological disorders (De Keyser et al., 2008). Impaired astrocytic modulation of the neuronal energy metabolism has been postulated as a candidate for the etiology of ADHD (Todd and Botteron, 2001). In addition, a recent study has

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demonstrated that an astrocyte-specific perturbation of SynCAM induces ADHD-like behavioral symptoms in mice (Sandau et al., 2012). This implies that impaired communication between astrocytes and neurons may cause ADHD-related symptoms.

Astrocytosis refers to an abnormal increase in the number of reactive astrocytes resulting from the death of nearby neurons. Reactive astrocytes are distinguished by their swollen cell body and altered expression of various proteins, including glial fibrillary acidic protein (GFAP), vimentin, and glutamine synthetase (Eid et al., 2004; Halassa and Haydon, 2010). The expression of glutamine synthetase, a key enzyme that mediates the conversion of glutamate into glutamine, is decreased in patients with epileptic seizures (Eid et al., 2004), which indicates that impaired astrocytic function may cause abnormal neuronal activity.

G protein-coupled receptor kinase-interacting protein-1 (*GIT1*) is a multifunctional signaling adaptor (Hoefen and Berk, 2006; Premont et al., 1998) associated with ADHD (Won et al., 2011). *Git1*$^{-/-}$ mice display an ADHD-like phenotype, including abnormal theta rhythms on EEG, hyperactivity, and impaired recognition memory, that is normalized by amphetamine treatment (Won et al., 2011). However, unlike other animal models of ADHD, *Git1*$^{-/-}$ mice do not have altered expression of tyrosine hydroxylase, implying that the ADHD-like behavioral manifestations of *Git1*$^{-/-}$ mice are not mediated by deficits in dopamine. Nonetheless, a psychostimulant that modulates dopamine release restores key behavioral features in *Git1*$^{-/-}$ mice, suggesting crosstalk between dopaminergic modulation and the general synaptic defects that are exhibited in these mice.

In the present study, we performed immunohistochemical analysis in the brains of *Git1*$^{-/-}$ mice to further understand the etiology of ADHD-like behavior. We found severe astrocytosis in the basal ganglia and thalamus, including the GP and thalamic reticular nucleus (TRN). Astrocytes have been identified as active players in synaptogenesis and synaptic transmission in the tripartite synapse (Volterra and Meldolesi, 2005); therefore, we examined neuronal markers in the astrocytosis-affected regions. These brain regions also exhibited altered expression of inhibitory presynaptic molecules, including parvalbumin and GABA. These results suggest that abnormalities in the basal ganglia circuit of *Git1*$^{-/-}$ mice are associated with the ADHD-like phenotype. Dopamine plays a crucial role in modulating basal ganglia circuit activity; therefore, these results provide mechanistic insights into how psychostimulants exert their effect on *Git1*$^{-/-}$ mice.

MATERIALS AND METHODS

Git1$^{-/-}$ mice

Git1$^{-/-}$ mice were generated as described previously (Won et al., 2011). Mice at 4 weeks and 2-3 months of age were used for immunohistochemical analysis. Experiments were performed in accordance with the guidelines of the Animal Welfare Committee of KAIST, Korea.

Antibodies

Antibodies against GluR2 (1:1000; #1195) were previously generated in our laboratory (Kim et al., 2009). The following antibodies were purchased: GFAP (1:1000), GABA (1:1000; Abcam); Iba1 (0.5 $\mu g \cdot ml^{-1}$; Wako); vGlut1 (2 $\mu g \cdot ml_{-1}$), vGAT (1:500; Synaptic Systems); NeuN (2.5 $\mu g \cdot ml^{-1}$), GAD67 (2 $\mu g \cdot ml^{-1}$), glutamine synthetase (2 $\mu g \cdot ml^{-1}$), parvalbumin (1:1000; Millipore); Bassoon (1:1000; Stressgen). The optimal concentration of antibodies was determined according to the manufacturer's recommended protocols.

Immunohistochemistry

Brains were isolated from adult mice (4 weeks or 2-3 months old) after cardiac perfusion (4% paraformaldehyde). Following post-fixation for 12 h, 50-μm sections were obtained using a vibratome (Leica). Sections were washed 3 times with PBS for 10 min, permeabilized with 0.5% Triton X-100 for 30 min, blocked with 5% bovine serum 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 quantitative analysis, images were captured with a confocal microscope (63× and 20× objectives; Leica Microsystems) and analyzed using Metamorph (Molecular Devices). We measured the strength of immunoreactivity from both the cell soma and processes.

RESULTS

Regional specificity of astrocytosis in the brains of *Git1*$^{-/-}$ mice

We hypothesized that the ADHD-like symptoms in *Git1*$^{-/-}$ mice may involve local changes in specific brain circuits. Intriguingly, we found significant increases in the number of GFAP-positive astrocytes (astrocytosis) in specific brain regions of 2-3-month-old *Git1*$^{-/-}$ mice. These regions included the GP and TRN, with the strongest increase (approximately 7 fold) in the GP (Figs. 1A and 1B). This astrocytosis was also evident in younger (4 week old) *Git1*$^{-/-}$ brains (Fig. 2A), suggesting that it may have a developmental origin. Milder astrocytosis was observed in thalamic and cortical regions at 2-3 months (Figs. 1A and 1B), whereas astrocytosis was absent in the striatum, amygdala, and hippocampus (Fig. 3).

This feature could represent reactive astrocytosis, which is often caused by neuronal damage or loss and can lead to neuroinflammation. However, the number of neurons and microglia were unchanged in the affected regions, including the GP and TRN (Figs. 1C and 4). In addition, glutamine synthetase, which is typically downregulated during reactive astrocytosis (Eid et al., 2004; Ortinski et al., 2010), was unchanged or increased in these regions (Fig. 5). This excluded the possibility of neuroinflammation and reactive astrocytosis.

Enhanced GABA signals in the globus pallidus and their colocalization with astrocytes

Astrocytes play an active role in neuronal transmission in the tripartite synapse model (Volterra and Meldolesi, 2005). There was no sign of neuroinflammation in the regions showing severe astrocytosis of *Git1*$^{-/-}$ mice (Figs. 1C and 4); therefore, we examined any astrocytosis-induced changes in neuronal or synaptic morphology in the GP and TRN using immunohistochemistry. We found a decrease in parvalbumin, a marker of fast-spiking interneurons, in the GP but not the TRN of *Git1*$^{-/-}$ mice (Fig. 6). This suggested that inhibitory inputs from parvalbumin-positive interneurons in the GP are decreased in these mice.

Notably, there was a significant increase in expression of the inhibitory neurotransmitter, GABA, in the GP, but not the TRN (Figs. 6A-6D). In addition, GABA was colocalized with GFAP-positive astrocytes but not Iba1-positive microglia in both 2-3-month-old and 4-week-old *Git1*$^{-/-}$ mice (Figs. 2B and 7). This suggested that the observed astrocytosis may be associated with abnormally enhanced GABA levels in the GP.

In addition, two thalamic regions (ventrobasal and ventrolateral) showed reduced signals of parvalbumin (Figs. 8C-8F); however,

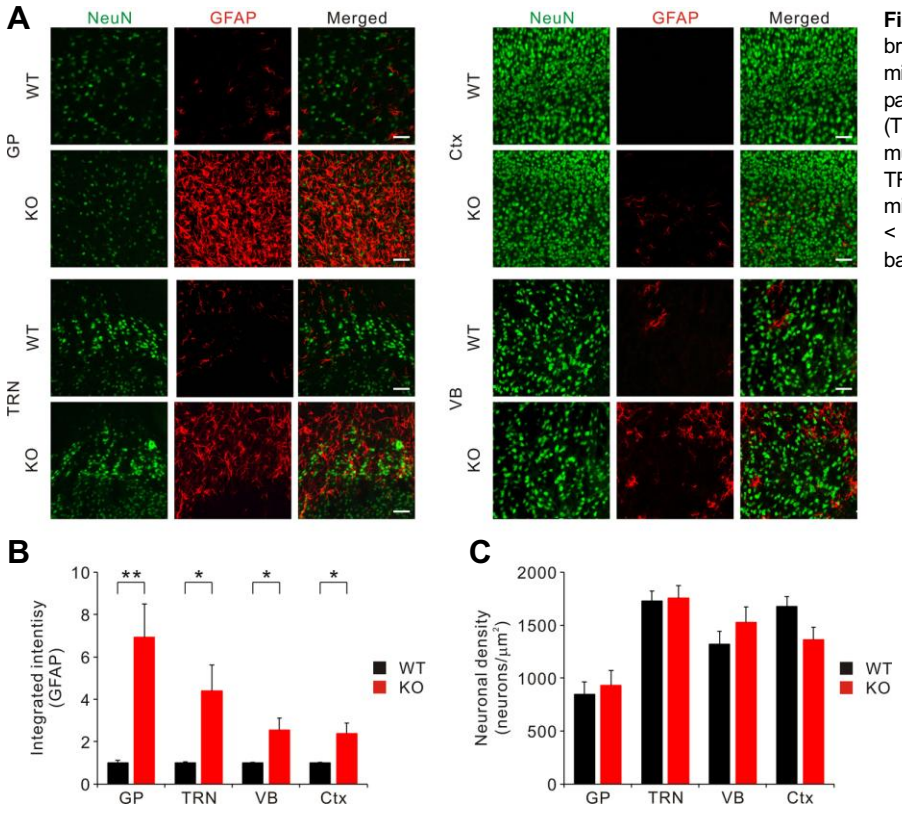

Fig. 1. Enhanced astrocytosis in specific brain regions of *Git1*$^{-/-}$ mice. (A, B) *Git1*$^{-/-}$ mice display astrocytosis in the globus pallidus (GP), thalamic reticular nucleus (TRN), cortex (Ctx), and ventrobasal thalamus (VB). (C) Neuronal densities in the GP, TRN, Ctx, and VB are not affected in *Git1*$^{-/-}$ mice. $n = 4$ slices from 4 mice (WT, KO). $^{*}P < 0.05$, $^{**}P < 0.01$; Student's *t*-test. Scale bar, 63 μm.

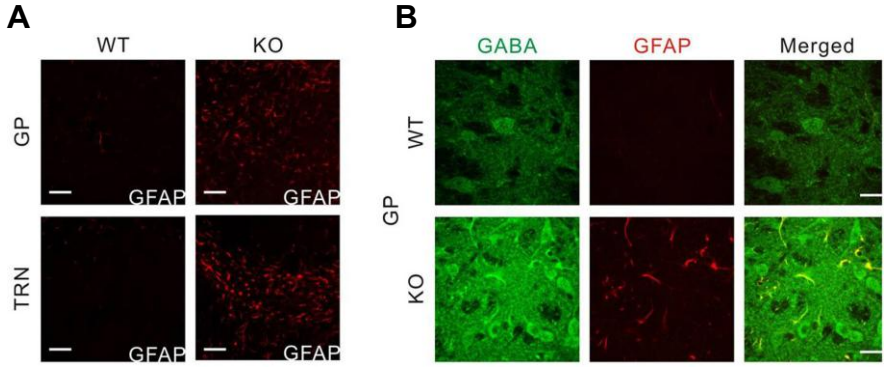

Fig. 2. Abnormally increased astrocytosis in the globus pallidus (GP) of 4 week old *Git1*$^{-/-}$ mice. (A) Severe astrocytosis occurs in the GP and thalamic reticular nucleus (TRN) of 4 week old *Git1*$^{-/-}$ mice. (B) Increased GABA is colocalized with GFAP in the GP. Scale bar, 20 μm.

no changes were observed in the striatum, including the caudate nucleus and putamen (Figs. 8A and 8B). Taken together, the astrocytosis and immunohistochemical data suggested that the GP, and related brain regions involved in the basal ganglia pathway, may have functional defects in *Git1*$^{-/-}$ mice.

DISCUSSION

We have reported severe astrocytosis in specific brain regions of *Git1*$^{-/-}$ mice. Astrocytosis was observed in the prefrontal cortex, GP, ventrobasal thalamus, and TRN, but not other brain regions. These brain regions displayed altered expression of synaptic markers and neurotransmitters, including reduced parvalbumin and increased GABA. These data indicate abnormal functioning of the basal ganglia pathway (cortico-striato-pallido-thalamic) in *Git1*$^{-/-}$ mice.

The basal ganglia comprise a collection of subcortical nuclei that are associated with modulation of motor activities, and their dysfunction is linked to motor defects in both Parkinson's and Huntington's disease (DeLong and Wichmann, 2007; Graybiel, 2000; Kravitz et al., 2010). Due to the severe astrocytosis and increased GABA levels in the GP of *Git1*$^{-/-}$ mice, we speculate that defects in the cortico-striato-pallido-thalamic circuit may contribute to their ADHD-like symptoms. In addition, this converges with the well-established dopamine deficit theory and excitatory/inhibitory (E/I) imbalance in *Git1*$^{-/-}$ mice and provides

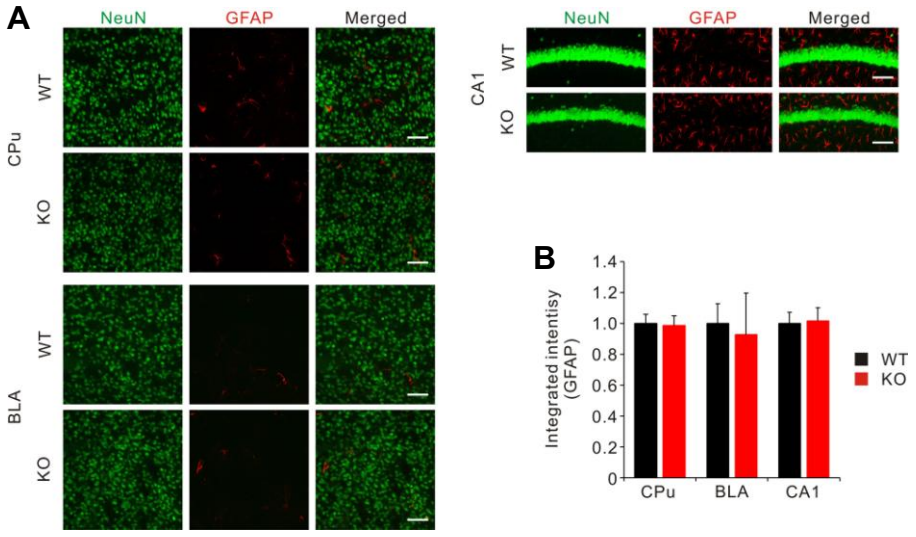

Fig. 3. No astrocytosis is found in the striatum (CPu), basolateral amygdala (BLA), and hippocampal CA1 region (CA1) of *Git1*$^{-/-}$ mice. The results in (A) were quantified in (B). *n* = 4 slices from 4 mice (WT, KO). Scale bar, 63 μm.

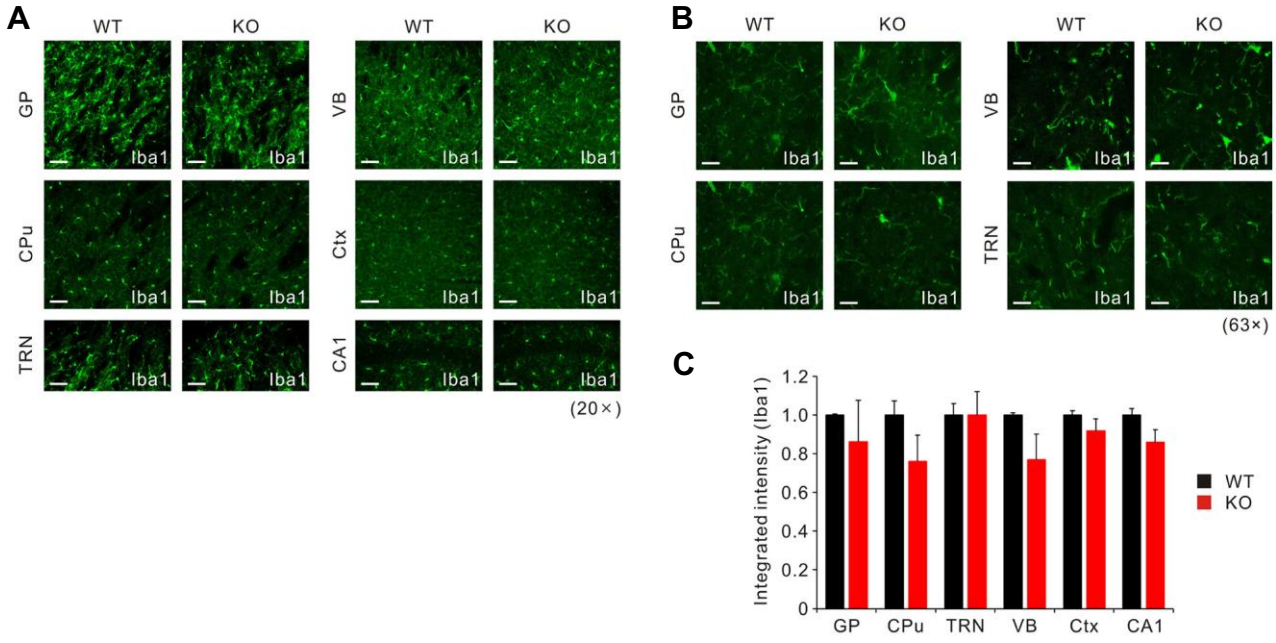

Fig. 4. Iba1 expression, a marker for microglia, is not changed in *Git1*$^{-/-}$ mice when compared with wild-type controls (WT). (A-C) Iba1 immunostaining in the globus pallidus (GP), striatum (CPu), thalamic reticular nucleus (TRN), ventrobasal thalamus (VB), cortex (Ctx), and CA1 region of the hippocampus (CA1) at 20× (A) and 63× (B). The results in (A) were quantified in (C). *n* = 4 slices from 4 mice (WT, KO). Scale bar, 63 μm (A) and 20 μm (B).

mechanistic insights on how a psychostimulant can rescue key behavioral characteristics in *Git1*$^{-/-}$ mice, which do not exhibit dopaminergic deficits. In line with this, anatomical and functional defects in the GP have been reported to be associated with ADHD (Aylward et al., 1996; Castellanos et al., 1996; Durston et al., 2003; Overmeyer et al., 2001; Swanson et al., 1998). Moreover, the thalamus, the terminal region of basal ganglia circuitry, has been implicated in ADHD and attention (Biederman, 2005; Ivanov et al., 2010).

Astrocytes are a well-known source of glutamine for neurons, which regulates their excitability *via* reuptake of excessive synaptic glutamate (Killeen et al., 2013). This glutamate is converted to glutamine by glutamine synthetase, which lowers the level of extracellular glutamate (Russell et al., 2006). Increased glutamine synthetase in the GP of *Git1*$^{-/-}$ mice lowers the glutamate level in the synapse, which may result in the overall downregulation of GP activity. In addition, previous studies have shown that inhibition of glutamine synthetase causes decreased inhibitory transmission and GABA release in neurons (Liang et al., 2006), while glutamatergic transmission is

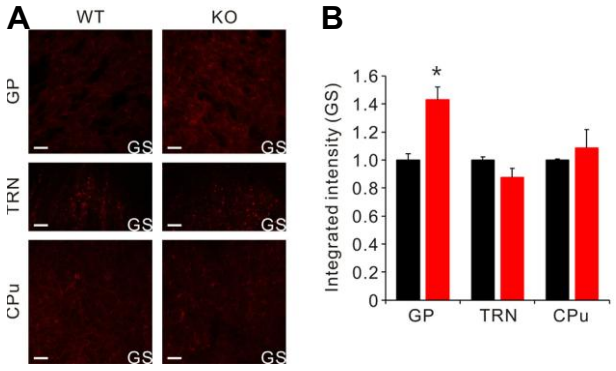

Fig. 5. Glutamine synthetase (GS) expression is increased in the globus pallidus (GP) but unchanged in the thalamic reticular nucleus (TRN) and striatum (CPu). (A, B) Representative images in (A) were quantified in (B). n = 4 slices from 4 mice (WT, KO). $P < 0.05$; Student's t-test. Scale bar, 63 μm.

unaffected (Kam and Nicoll, 2007). These results suggest that neurons in the GP of *Git1*$^{-/-}$ mice may show decreased glutamatergic transmission and increased GABAergic transmission, the latter of which was reflected by increased GABA in the GP in this study.

Increased GABA in the GP may arise *via* GABA release from increased astrocytes; GABA release has been reported from cerebellar glial cells (Lee et al., 2010). GABA released from astrocytes may affect the overall activity in the GP, leading to increased tonic inhibition in this brain region. Alternatively, astrocytosis may occur in response to altered inhibitory neurotransmission in these regions.

Although the source of the increased GABA is unclear, it is likely to significantly inhibit GP GABAergic neurons in *Git1*$^{-/-}$ mice, thus weakening the inhibition of their target neurons in the endopeduncular nucleus and TRN. These changes would enhance the inhibitory influence of GABAergic afferents from the endopeduncular nucleus and TRN on the thalamus, an important sensory relay center that mediates sensory gating and attention.

Our finding of increased GABA in the GP is in contrast to a previous study that shows decreased inhibition in the hippocampus of *Git1*$^{-/-}$ mice, which results in an imbalance of excitatory and inhibitory synaptic transmission (Won et al., 2011). This suggests that a single genetic modification may cause different physiological phenotypes depending on the affected neuronal circuit. Recently, it has been reported that *Neuroligin-3* knock-in mice, a model of autism, show decreased inhibition in striatal medium spiny neurons (Rothwell et al., 2014). In addition, previous studies in the same mouse model have reported increased inhibitory transmission in the cortex and hippocampus (Foldy et al., 2013; Tabuchi et al., 2007). These studies,

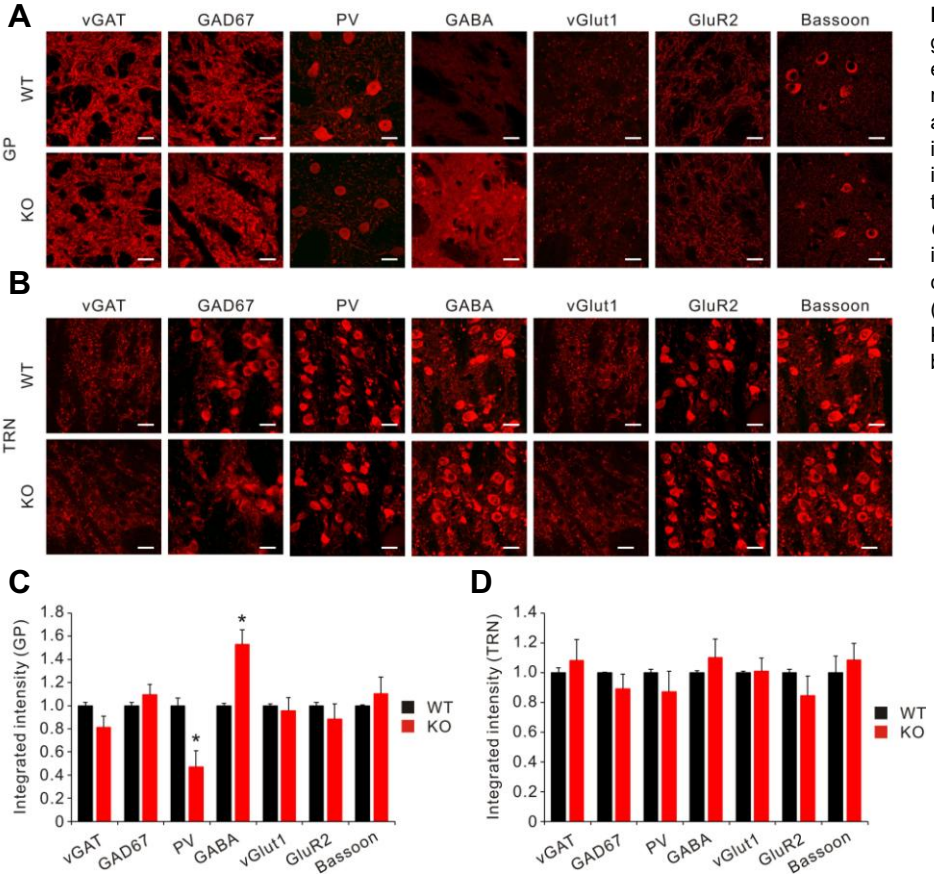

Fig. 6. Enhanced GABA levels in the globus pallidus (GP). (A, C) Reduced expression of parvalbumin (PV), a marker for fast-spiking interneurons, and increased expression of the inhibitory neurotransmitter GABA are seen in the GP of *Git1*$^{-/-}$ mice. (B, D) The thalamic reticular nucleus (TRN) of *Git1*$^{-/-}$ mice does not have differences in neuronal or synaptic markers when compared with wild-type controls (WT). n = 4 slices from 4 mice (WT, KO). *$P < 0.05$; Student's t-test. Scale bar, 20 μm.

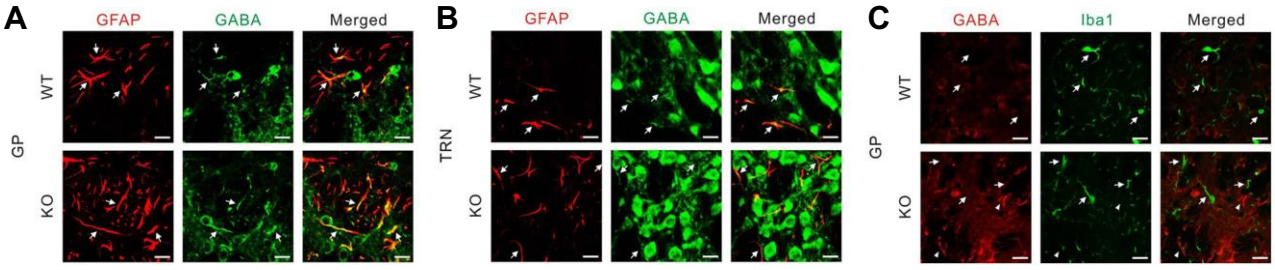

Fig. 7. GABA expression colocalizes with astrocytes but not microglia. (A, B) GABA immunostaining in the globus pallidus (GP, A) and thalamic reticular nucleus (TRN, B) colocalizes with the astrocytic marker, GFAP (indicated by arrows). (C) GABA does not colocalize with the microglial marker, Iba1, in the GP. GABA negative and Iba1 positive staining is indicated by arrows. GABA positive and Iba1 negative staining is indicated by arrowheads. Scale bar, 20 μm.

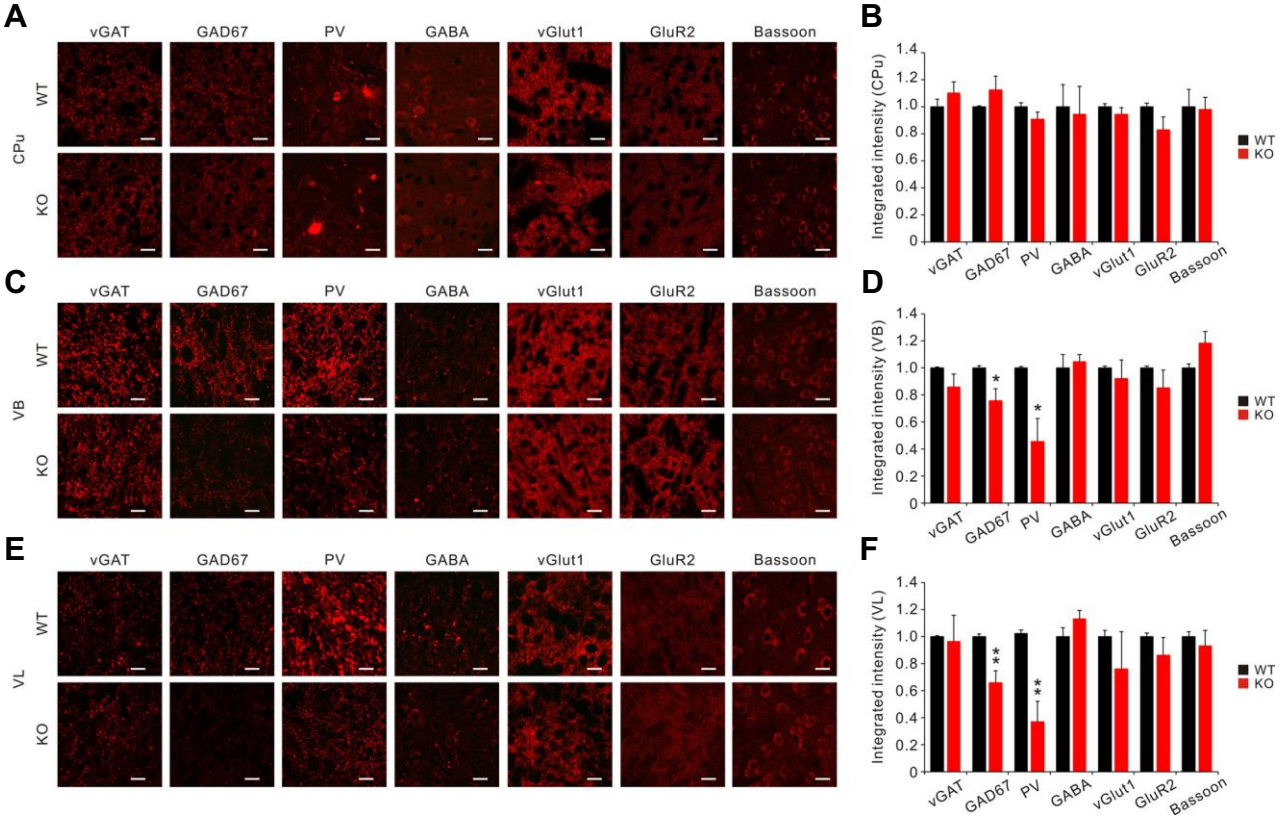

Fig. 8. Reduced inhibitory markers in the thalamus of *Git1*$^{-/-}$ mice. (A, B) Most synaptic and neuronal markers are unaltered in the striatum (CPu) of *Git1*$^{-/-}$ mice. The results in (A) were quantified in (B). (C-F) Reduced levels of parvalbumin (PV), a marker for fast-spiking interneurons, and GAD67, a marker for inhibitory synapses, in the ventrobasal (VB, C, D) and ventrolateral (VL, E, F) thalamus of *Git1*$^{-/-}$ mice. The results in (C) were quantified in (D), and the results in (E) were quantified in (F). n = 4 slices from 4 mice (WT, KO). $P < 0.05$; Student's t-test. Scale bar, 20 μm.

together with the data presented here, suggest that one genetic mutation or deletion can have diverse physiological effects in a circuit-specific manner.

It is unclear why severe astrocytosis was observed in specific brain regions and how this affected parvalbumin-positive GABAergic interneuron functioning. The astrocytosis observed differed from conventional astrocytosis, which is associated with neuronal loss and neuroinflammation, suggesting that it occurred in a cell-autonomous manner. *GIT1* plays a critical role in cell migration and polarization (Penela et al., 2014); therefore, an increase in the number of astrocytes in specific brain regions may be the result of abnormal cell migration. Moreover, the small GTPase, Rac1, is required for cell polarization, directed migration (Fukata et al., 2003), and regulation of astrocytic migration (Etienne-Manneville and Hall, 2001). miR-509-3p, an miRNA targeting CDK2, Rac1, and PIK3C2A, inhib-

its cell proliferation and migration, which suggests a role for Rac1 in cell migration (Yoon et al., 2014). Rac1 activity is downregulated in the brains of *Git1*$^{-/-}$ mice (Won et al., 2011); therefore, the migration of astrocytes may be impaired, resulting in abnormal astrocytosis in specific brain regions. Alternatively, this may be caused by increased proliferation of astrocytes because GIT1 plays a role in contact inhibition of proliferation (Liu et al., 2010). Currently, the role of GIT1 in astrocytes has not been elucidated and warrants future investigation.

It is important to address how astrocytosis might affect behavioral manifestations in *Git1*$^{-/-}$ mice. Several lines of evidence suggest links between hyperactivity and astrocytes: 1) the perturbation of the glutamate metabolism by the deletion of the glial glutamate transporter, GLAST, causes schizophrenia-like novelty-induced hyperactivity (Karlsson et al., 2008); 2) astrocytosis is detected in a repetitive mild traumatic brain injury (rmTBI) animal model that exhibits hyperactivity (Mannix et al., 2014); and 3) the ablation of D1 dopamine receptor expressing cells causes astrocytosis in the striatum, which has been connected to hyperactivity (Gantois et al., 2007). However, the astrocytosis reported in these studies is hypothesized to result from neuronal cell death, which initiates reactive astrocytes. This reactive astrocytosis is distinguishable from the astrocytosis detected in *Git1*$^{-/-}$ mice because they show normal neuronal density and unaltered or increased expression of astrocytic glutamine synthetase. Therefore, the etiological role of non-reactive astrocytosis in *Git1*$^{-/-}$ mice may be different from these models. It will be a challenging topic to investigate how this affects the microcircuit and behavioral perturbations in *Git1*$^{-/-}$ mice.

In conclusion, we have observed severe astrocytosis and an altered expression of GABA and parvalbumin in the brain regions related to basal ganglia circuitry in *Git1*$^{-/-}$ mice. The resulting dysfunction may be a neural correlate of ADHD-like behavioral symptoms reported in these mice. This may form a nexus with the previously proposed dopamine hypothesis of ADHD because dopamine is closely linked to basal ganglia function (DeLong and Wichmann, 2007). Hence, we postulate an extended hypothesis for ADHD pathophysiology: the basal ganglia dysfunction theory in ADHD.

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