

Glutamine Supplementation Ameliorates Chronic Stress-induced Reductions in Glutamate and Glutamine Transporters in the Mouse Prefrontal Cortex

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Chronic immobilization stress (CIS) induces low levels of glutamate (Glu) and glutamine (Gln) and hypoactive glutamatergic signaling in the mouse prefrontal cortex (PFC), which is closely related to the Glu-Gln cycle. A Gln-supplemented diet ameliorates CIS-induced deleterious changes. Here, we investigated the effects of CIS and Gln supplementation on Glu-Gln cycle-related proteins to characterize the underlying mechanisms. Using the CIS-induced depression mouse model, we examined the expression of 11 proteins involved in the Glu-Gln cycle in the PFC. CIS decreased levels of glutamate transporter 1 (GLT1) and sodium-coupled neutral amino acid transporter (SNAT) 1, SNAT2, SNAT3, and SNAT5. Gln supplementation did not affect the non-stressed group but significantly increased GLT1 and SNATs of the stressed group. By immunohistochemical analysis, we confirmed that SNAT1 and SNAT2 were decreased in neurons and GLT1, SNAT3, and SNAT5 were decreased in astrocytes in the medial PFC of the stressed group, but Gln-supplemented diet ameliorated these decrements. Collectively, these results suggest that CIS may cause depressive-like behaviors by decreasing Glu and Gln transportation in the PFC and that a Gln-supplemented diet could prevent the deleterious effects of CIS.

Key words: Depressive disorder, Stress, Glutamate, Glutamine, Prefrontal cortex

INTRODUCTION

Glutamate (Glu) is a major excitatory neurotransmitter in the

central nervous system (CNS) and is involved in the detoxification of ammonia and synthesis of peptides and proteins. Due to its multiple functions and extremely high intracellular concentration in the brain (1~10 mM), Glu must be tightly regulated to limit extracellular levels to ensure optimal neurotransmission and prevent potential excitotoxicity [1, 2]. However, transporter-mediated uptake and recycling of Glu via the Glu-Glutamine (Glu-Gln) cycle is sensitive to stress and glucocorticoids. Emerging evidence has shown that disturbed glutamatergic neurotransmission in the CNS is the main cause for stress-induced psychiatric disorders

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including major depressive disorder (MDD) [1, 3].

Astrocytes are the major glial cells and play a crucial role in regulating glutamatergic signaling via the Glu-Gln cycle [4-8]. The central functions of astrocytes include rapid elimination of Glu released from presynaptic neurons and mediating neurotransmitter metabolism via the Glu-Gln cycle, enabling astrocytes to control synaptic activity. However, stress and glucocorticoids (mainly corticosterone in rodents) increase extracellular Glu levels, thus disrupting optimal collaboration between astrocytes and neurons to produce desired functions [1, 4].

A variety of studies in MDD patients and depressive rodent models have shown altered glutamatergic neurotransmission in the brain, such as low glutamatergic synaptic activity, reduced Glu and Gln levels, and changes in expression levels or activities of the Glu-Gln cycle proteins [3, 9-12]. Disturbance of the Glu-Gln cycle via L- α -amino adipic acid (an astrocyte-specific toxin), methionine sulfoximine (a Gln synthetase (GS) inhibitor), and α -methyl-amino-isobutyric acid (a blocker of neuronal Gln transporters) leads to depressive behaviors and decreased Glu and Gln levels in the prefrontal cortex (PFC) of rodents [3, 13, 14]. Moreover, direct infusion or supplementation of Gln reverses the depressive behaviors and Glu-Gln cycle impairments, implying that disrupted glutamatergic signaling and neuronal Gln deficiency mediate depression [3, 12, 14].

We recently demonstrated that chronic immobilization stress (CIS) causes depressive behaviors by reducing GS activity and Glu and Gln levels, ultimately leading to hypoactive glutamatergic neurotransmission in the medial PFC (mPFC) of mice; however, a Gln-supplemented diet reversed these effects [3]. To further investigate how CIS affected Glu and Gln levels and how exogenous Gln restored it, we evaluated changes in the expression levels of Glu-Gln cycle proteins in the PFC caused by CIS and Gln supplementation.

MATERIALS AND METHODS

Animals

Male 7-week-old C57BL/6 mice (Koatech, Pyeongtaek, Republic of Korea) were habituated for 1 week before experiments in a specific pathogen-free animal facility at the School of Medicine, Gyeongsang National University. Mice were individually housed at a constant temperature (22~24°C) under a 12-h light/dark cycle (lights on at 6 am) with free access to laboratory chow and water. Gln-supplemented diets (150 mg/kg) were fed to the Gln group, and calorie-balanced normal chow diets were fed to the control group (Uni Faith, Seoul, Republic of Korea) during the entire period of the experiments as previously described [3]. Mice were ran-

domly grouped using a computer-generated list according to body weight. Control (CTL) and control-glutamine (CTL+Gln) groups were caged with or without Gln supplementation. Stress (STR) and stress-glutamine (STR+Gln) groups were exposed to stress with or without Gln supplementation. Animal use procedures were performed in accordance with the National Institutes of Health guidelines and an approved protocol (GNU-140225-M0012) by the Gyeongsang National University Institution Animal Care & Use Committee.

Chronic immobilization stress

CIS was carried out as previously described [15]. Briefly, mice were repeatedly placed in a restrainer for 2 h/day (14:00~16:00) for 15 days under 200 lux light conditions. Body weight and food intake were measured every other day. After CIS, one batch of mice was subjected to depressive behavioral tests. The other batch was sacrificed by decapitation at 9:00~11:00 am and used for molecular analyses such as plasma corticosterone analysis and expression level analyses of Glu-Gln cycle proteins, without undergoing behavioral tests (Fig. 1A).

Behavioral tests

The sucrose preference test (SPT) was performed with some modifications as previously described to identify symptoms of anhedonia [14]. Briefly, mice were habituated for 48 h with a palatable sucrose solution (0.1 M), followed by a 24-h water deprivation period and a 6-h exposure to two identical bottles, one filled with sucrose solution and the other with water. Sucrose solution and water consumption were determined for 6 h. The sucrose preference was represented as the ratio of sucrose-to-water consumption.

The tail suspension test (TST) was conducted as previously described with some modifications [3]. Mice were individually suspended by the tail to a horizontal bar (the distance from the floor was 30 cm) using clear tape (distance from the tip of the tail was approximately 1 cm). The duration of immobility during the 6-min test session was recorded and analyzed by an animal behavior video analysis program (EthoVision; Noldus Information Technology, Wageningen, The Netherlands).

Plasma corticosterone level analysis

Mouse blood was collected in vacutainers containing K3EDTA. Plasma was isolated by centrifugation at 1,000 \times g, at 4°C for 10 min. The samples were stored at -80°C until use. Quantification of plasma corticosterone (CORT) levels was carried out using a CORT enzyme-linked immunosorbent assay kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's protocol.

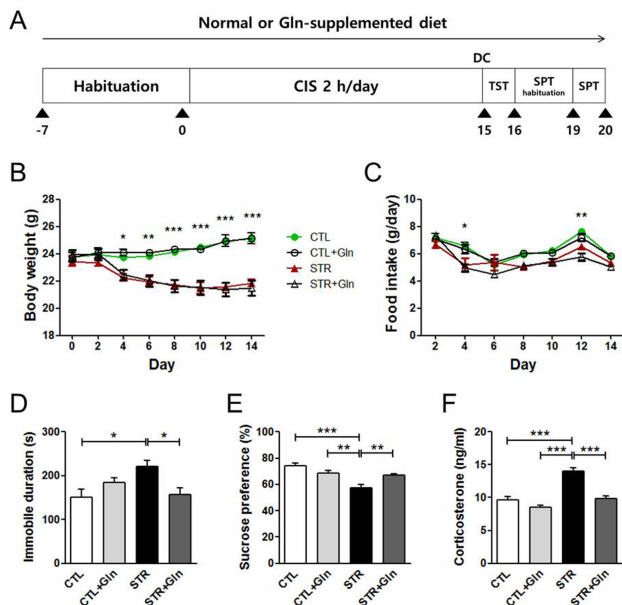


Fig. 1. Glutamine (Gln) supplementation inhibited the deleterious effects of chronic immobilization stress (CIS). (A) Experimental schedule. One batch was sacrificed by decapitation after CIS for molecular analysis, and the other was used for behavioral tests. Body weight (B) and food intake (C) were recorded every 2 days for 2 weeks from day 0. (D and E) The effects of CIS and Gln on immobility duration during the tail suspension test (D) and sucrose preference (E) were analyzed after CIS. (F) Plasma corticosterone levels were analyzed after CIS without behavioral tests. The bars represent the means±SEMs. *p<0.05, **p<0.01, ***p<0.001 in the two-way (B and C) or one-way (D~F) analysis of variance test with Newman-Keuls multiple comparison test (n=5~7).

Western blot analysis

Crude proteins were extracted from the PFC by homogenizing with glass beads and RIPA buffer (Elpis-Biotech, Daejeon, Republic of Korea) using a Bullet Blender (Next Advance, New York, NY, USA), followed by sonication for 2 min and centrifugation at 12,000×g at 4°C for 10 min. Protein samples (10 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk or 3% bovine serum albumin in Tris-buffered saline with Tween (50 mM Tris, 150 mM NaCl, 0.1% or 1% Tween-20, pH 7.4) for 2 h and incubated overnight at 4°C with the following primary antibodies: PC (1:2,000, SC373937; Santa Cruz Biotechnology, Dallas, TX, USA), GDH [1:5,000, SC160383 (GDH1/2); Santa Cruz Biotechnology], GLT1 (1:1,000, SC7760; Santa Cruz Biotechnology), GS (1:10,000, MAB302; EMD Millipore, Temecula, CA, USA), SNAT3 (1:1,000, ab211516; Abcam, Cambridge, UK), SNAT5 (1:2,000, ab72717; Abcam), SNAT1 (1:1,000, SC67080; Santa Cruz Biotechnology), SNAT2 (1:1,000, SC166366; Santa Cruz Biotechnology), GLS2 (1:2,000, AB113509; Abcam), GAD1 (1:2,000, 5305; Cell Signal-

ing, Danvers, MA, USA), GAD2 (1:2,000, 3988; Cell Signaling), α-tubulin (1:10,000, T5168; Sigma-Aldrich, St. Louis, MO, USA), and β-actin (1:10,000, A5441; Sigma-Aldrich). After secondary antibody binding for 2 h with anti-rabbit IgG-horseradish peroxidase (HRP) (1:10,000, NCI1460KR; Thermo Fisher Scientific, Waltham, MA, USA) or anti-mouse IgG-HRP (1:10,000, NCI1430KR; Thermo Fisher Scientific) antibodies, immunoblot signals were detected using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific) and iBright FL1000 (Thermo Fisher Scientific). Band intensities were calculated using iBright analysis software (Thermo Fisher Scientific). Expression levels were normalized against the internal controls, α-tubulin or β-actin.

Immunohistochemistry (IHC)

IHC for GLT1 and SNATs were performed as previously described [3] with some modifications. Twenty-four hours after the last stress, mice were deeply anesthetized with avertin and perfused with phosphate-buffered saline (pH 7.4) and 4% paraformaldehyde. The brains were collected, postfixed, sectioned at 40-µm thickness, and incubated with an antibody (1:20~1:200) at room temperature or 4°C overnight. An anti-gial fibrillary acidic protein (GFAP) antibody (Z0334) (1:200; Dako, Glostrup, Denmark), anti-GS antibody (MAB302) (1:200; Merck Millipore, MA, USA), anti-NeuN antibody (MAB377) (1:200; Merck Millipore), and anti-GLS2 (AB113509) (1:200; Abcam) were used as cellular distribution markers. The slices were then incubated with Alexa Fluor 594- and 488-conjugated secondary antibodies (1:1,000; Invitrogen, Carlsbad, CA, USA). Digital images were captured using a spinning disk confocal microscope equipped with an Olympus Disk Spinning Unit (Olympus, Tokyo, Japan) and analyzed by ImageJ software (NIH).

Statistical analysis

Data are presented as means±SEMs. Student's *t*-tests were used to compare means between two groups. For multiple comparisons, one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison tests as post-hoc analysis was performed. A value of p<0.05 was considered statistically significant. Prism software, version 5.01 (GraphPad, La Jolla, CA, USA) was used for all statistical analyses. Experiments were repeated two or three times individually, and representative images are presented in the figures.

RESULTS

Gln lowers CIS-induced depressive behaviors and corticosterone levels

CIS decreased the body weight of the stress group (STR) and

Gln-supplemented STR group (STR+Gln), but there was no difference in the food intake between the tested groups (Fig. 1B and 1C). Gln supplementation did not affect body weight or food intake.

The STR group showed higher immobility in the TST and less sucrose preference compared with the CTL group (Fig. 1D and 1E), indicating that CIS induced depressive behaviors. Gln prevented these in the STR group (STR vs. STR+Gln), but no significant effect was observed in normal control mice (CTL vs. CTL+Gln). Moreover, basal plasma CORT levels also markedly increased after CIS, but they remained at the CTL level with Gln supplementation (Fig. 1F).

CIS and Gln supplementation altered the expression of Glu-Gln cycle proteins in the PFC

The effects of CIS and Gln on Glu-Gln cycle protein expression in the PFC were investigated by immunoblotting (Fig. 2). The expression level of the Glu transporter GLUT1 decreased after CIS. In addition, the Gln transporters SNAT2, SNAT3, and SNAT5 decreased in the PFC of CIS-subjected mice. Gln supplementation did not affect the non-stressed group (CTL+Gln). However, the expression levels of SNAT1, SNAT2, SNAT3, and SNAT5 in the STR+Gln group were significantly higher than those of the STR group (Fig. 2).

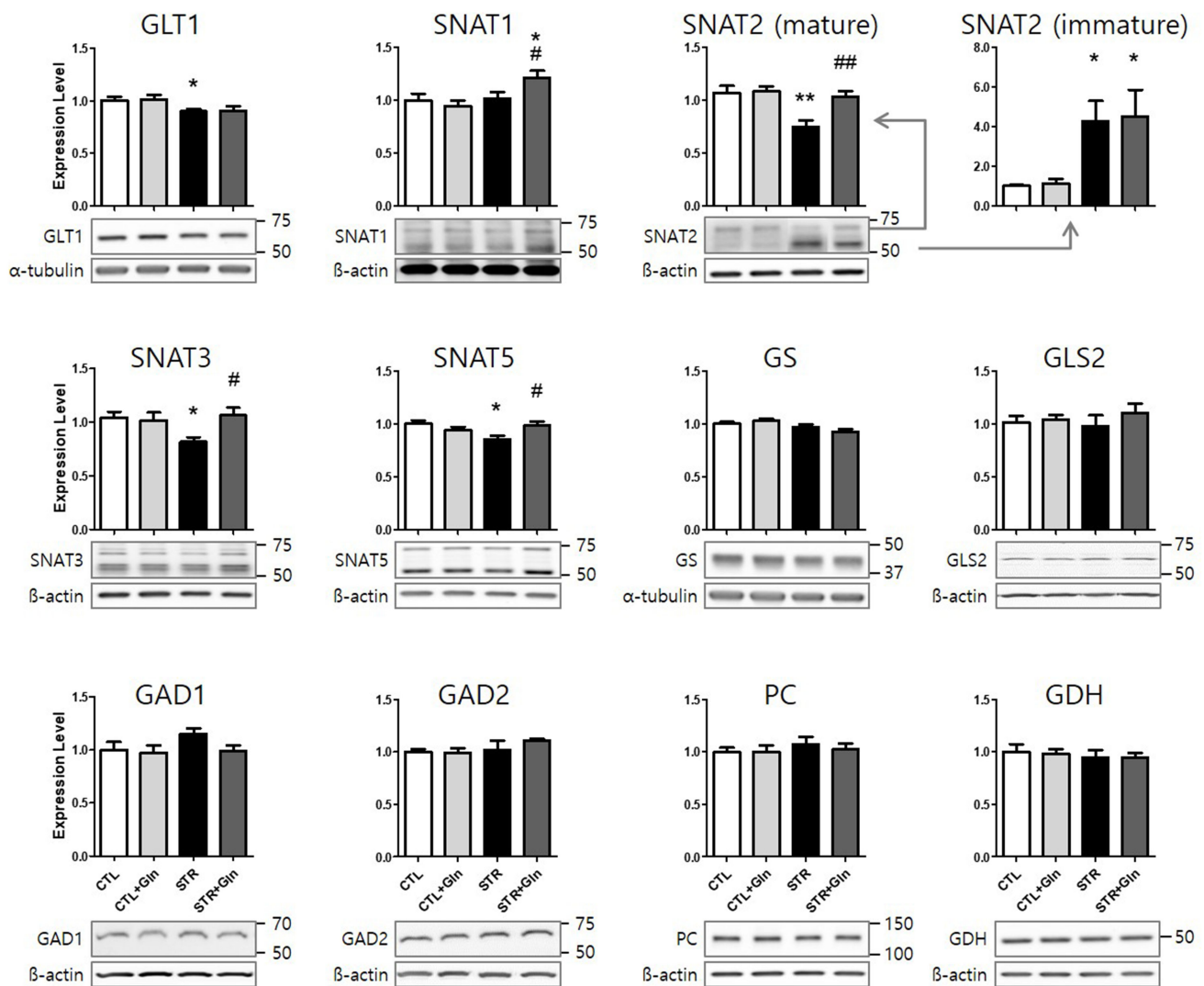


Fig. 2. Chronic immobilization stress (CIS) and glutamine supplementation induced expressional changes of the glutamate-glutamine (Glu-Gln) cycle in the prefrontal cortex. Bars represent the means±SEMs of relative expression levels from immunoblotting. *p<0.05, **p<0.01 (vs. CTL), #p<0.05, ##p<0.01 (vs. STR), one-way ANOVA with Newman-Keuls multiple comparison test as post-hoc analysis. GLUT1, glutamate transporter 1; SNATs, sodium-coupled neutral amino acid transporters; GS, glutamate synthetase; GLS2, glutaminase 2; GADs, glutamate decarboxylases; PC, pyruvate carboxylase; GDH, glutamate dehydrogenase. n=7~17. The figure shows a representative group. Molecular weights are marked on the sides of the immunoblots.

A mature glycosylated form (~60 kDa) and an immature form (non- or partially glycosylated, ~50 kDa) of SNAT2 [16] showed different expression patterns in response to CIS; the mature form decreased, whereas the immature form increased. The mature and

immature forms of the other Gln transporters (SNAT1, SNAT3, and SNAT5) showed similar expression patterns (Fig. 2).

To confirm the immunoblot results and cellular locations, expression level changes of GLT1 and SNATs in response to CIS and

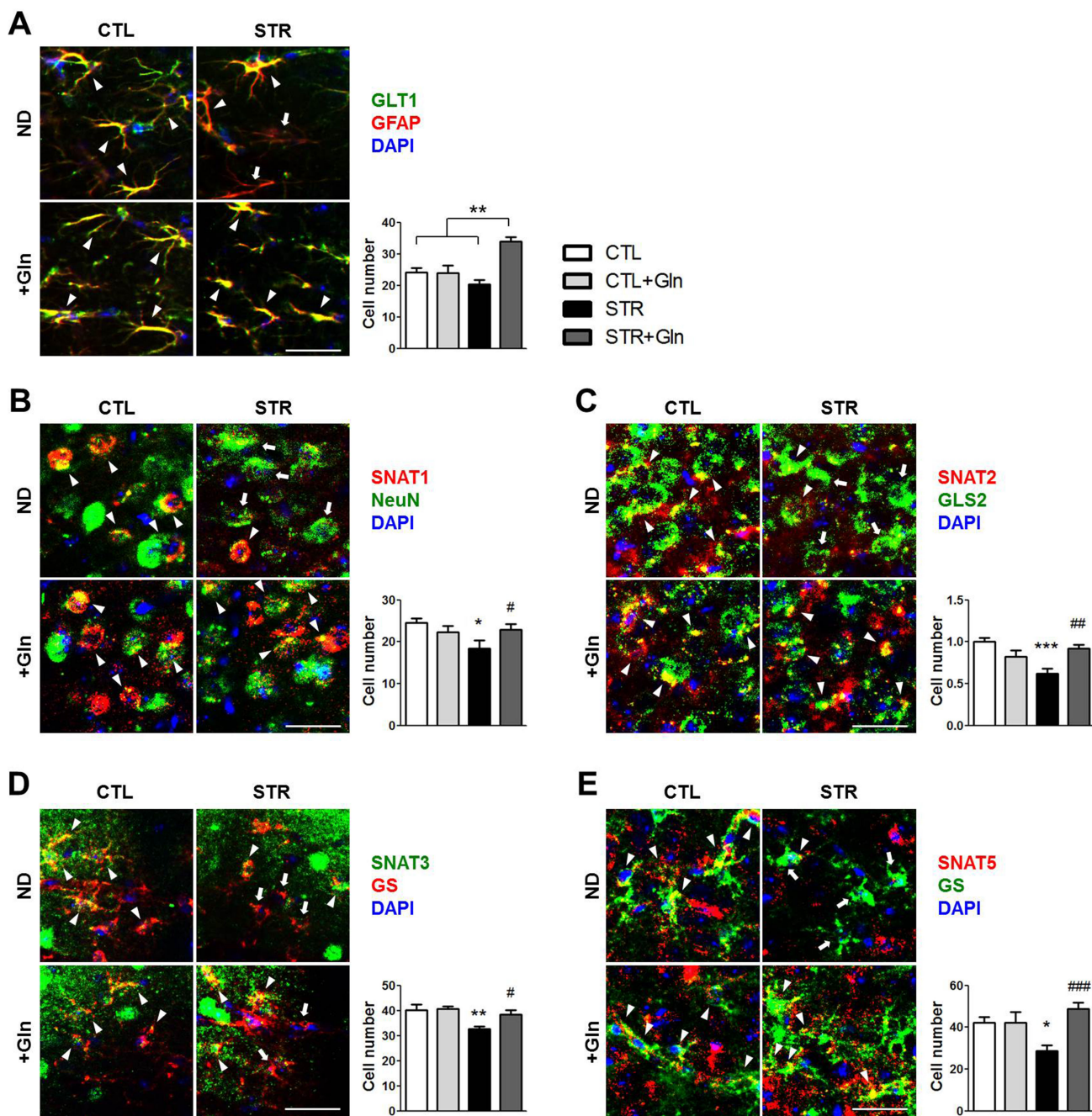


Fig. 3. Chronic immobilization stress (CIS) and glutamine supplementation induced expressional changes of GLT1 and SNATs in the medial prefrontal cortex. Representative mPFC immunohistochemistry images of (A) glutamate transporter GLT1 and (B–E) sodium-coupled neutral amino acid transporters (SNATs) merged with a cellular marker (GFAP and GS for astrocytes, NeuN and GLS2 for neurons). Scale bars: 25 μ m. Arrowheads indicate double-positive cells and arrows indicate cellular marker single-positive cells. Double-positive cell numbers are presented with the images ($n=12/\text{group}$, cell number/ 0.15 mm^2). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (vs. CTL), # $p<0.05$, ## $p<0.01$, ### $p<0.001$ (vs. STR), one-way ANOVA with Newman-Keuls multiple comparison test as post-hoc analysis.

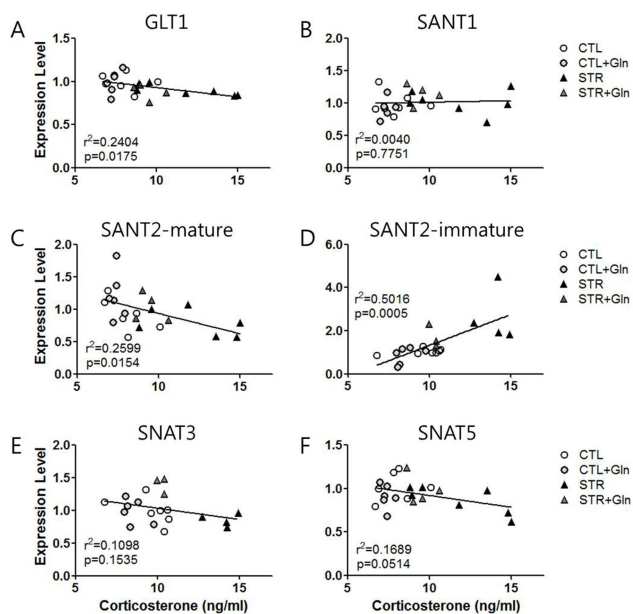


Fig. 4. Correlation analysis of plasma corticosterone and protein expression levels. The correlation coefficient (r^2) and correlation significance (p) are provided for each comparison in the figure. GLT1, glutamate transporter 1; SNATs, sodium-coupled neutral amino acid transporters.

Gln supplementation were analyzed by IHC in the mPFC (Fig. 3). Double-positive cells for each protein with neuronal or astrocytic marker were decreased by CIS, but not in the stressed group with Gln supplementation, supporting the immunoblot results. In addition, IHC showed a significant GLT1 induction by Gln (Fig. 3A) and SNAT1 reduction in the mPFC by CIS (Fig. 3B). These expression changes were not significant in the immunoblots performed with crude PFC protein samples.

GLT1, SNAT2, and SNAT5 expression levels correlate with plasma corticosterone concentrations

To assess whether CORT levels contribute to the regulation of Glu-Gln cycle proteins, we analyzed the correlation between plasma CORT and the Glu-Gln cycle protein expression levels in response to CIS (Fig. 4). GLT1 and mature and immature SNAT2 expression levels strongly correlated with plasma CORT levels (p -values near or <0.01). In addition, SNAT5 also showed a negative correlation with plasma CORT levels with a p -value of approximately 0.05, suggesting that the expression of GLT1, SNAT2, and SNAT5 might be associated with plasma CORT levels. SNAT1 and SNAT3 had weak or insignificant correlations with plasma CORT levels.

DISCUSSION

Reduced levels of Glu and Gln in the frontal cortex have been consistently reported in MDD patients [17-19]. In the brain, Gln is exclusively synthesized in astrocytes by GS and is involved in the Glu-Gln cycle to maintain glutamatergic neurotransmissions [20]. Our recent study showed that CIS reduced GS activity, Glu-Gln levels, and the spontaneous excitatory synaptic current (sEPSC) frequency of glutamatergic neurons in the mPFC, which was resulted in depressive behaviors [3]. Moreover, we showed that Gln supplementation prevented these deleterious changes induced by CIS, implying an antidepressant effect of Gln [3]. In the present study, we investigated the effects of CIS and Gln supplementation on Glu-Gln cycle-related protein levels to identify the underlying mechanisms that could cause Glu and Gln shortages in the PFC of a CIS-induced depression mouse model.

The effects of CIS and Gln on behavior and plasma CORT levels were similar to our previous studies [3, 14, 15], confirming that CIS induces depressive behaviors in mice and Gln supplementation prevents chronic stress-induced depression (Fig. 1). We found that GLT1, SNAT1, SNAT2, SNAT3, and SNAT5 were decreased in the PFC after CIS (Fig. 2, 3, and 5). However, Gln supplementation increased their levels under the stress condition. These results suggest that CIS disturbed the Glu-Gln cycle, possibly resulting in a shortage of Glu and Gln levels in astrocytes and neurons, whereas Gln supplementation ameliorated deleterious CIS-induced changes in the Glu-Gln cycle by facilitating translocation of Glu into astrocytes and Gln into neurons for glutamatergic signaling.

Previous studies have suggested low GLT1 in the brain (anterior cingulate cortex, left dorsolateral PFC, amygdala, and hippocampus) as a possible cause of depressive disorders [11, 21-24]. Blockade of GLT1 induced anhedonia and impaired spatial memory [25], whereas stimulation of GLT1 expression had antidepressant effects [26-30]. The decrease of astrocytic Glu uptake due to GLT1 reduction might lead to a shortage of Gln that is synthesized from Glu by GS in astrocytes.

However, Glu in the synaptic cleft would increase, at least at the early stage of GLT1 reduction, because Glu removal decreased. GLT1 is the predominant Glu transporter in the mature brain and accounts for ~95% of total Glu uptake in the forebrain [7, 31]. The reduction of GLT1 is therefore potentially neurotoxic and could affect glutamatergic signaling efficiency [11].

We reported that specific blocking of neuronal glutamine transportation mediated by SNAT1/2 caused depressive-like behaviors with low levels of Glu-Gln [14] and disturbed glutamatergic sEPSCs in response to exogenous Glu and Gln [3]. In the present study, we found decreased SNAT1/2 in the STR and increments

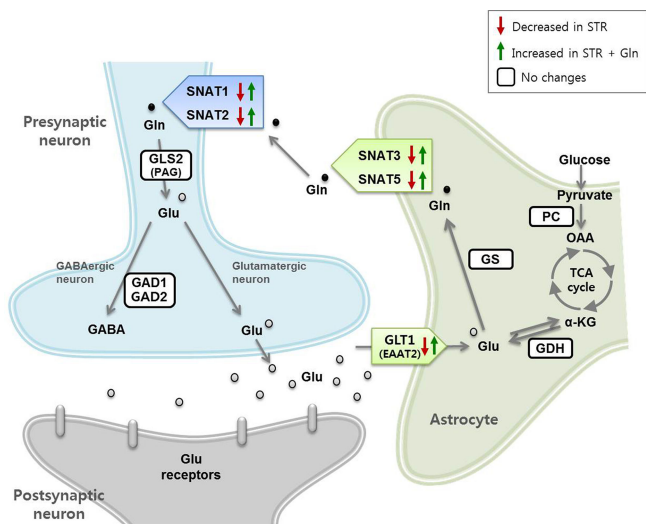


Fig. 5. The glutamate/glutamine cycle. High-affinity excitatory amino acid transporters (EAATs) of astrocytes primarily clear Glu in the synaptic cleft that is released from presynaptic neurons. GLT1 (glutamate transporter 1/EAAT2/SLC1A2) is the major regulator that transports Glu to astrocytes. In astrocytes, Glu is converted into Gln by glutamine synthetase (GS). Synthesized Gln is released from the astrocytes through sodium-coupled neutral amino acid transporters (SNAT3/SLC38A3 and SNAT5/SLC38A5). Released Gln is transported into neurons via SNAT1 and SNAT2 (SLC38A1 and SLC38A2) and converted back to Glu by phosphate-activated glutaminase (PAG or GLS1/2, mainly GLS2 in brain) for synaptic transmission of glutamatergic neurons. Glutamate is further transformed to γ -aminobutyric acid (GABA) by glutamic acid decarboxylases (GAD1 and GAD2) in the GABAergic neurons. Glu can be synthesized in the astrocytes from glucose via the tricarboxylic acid (TCA) cycle, followed by transamination of α -ketoglutarate (α -KG). Pyruvate carboxylase (PC) converts pyruvate to oxaloacetate that is converted to α -KG via the TCA cycle. Glutamate dehydrogenase (GDH) connects the TCA and Glu-Gln cycles by reversibly converting Glu into α -KG and vice versa. Expression changes by chronic immobilization stress (CIS) and Gln supplementation are marked by red and green arrows, respectively.

in the STR+Gln group (Fig. 2 and 3). These findings suggest that neuronal Gln transportation might be a crucial step for normal glutamatergic signaling and maintenance of normal behaviors during chronic stress, which also support our previous hypotheses [3, 14]. The functions of SNAT3 and SNAT5 in MDD pathogenesis remain unclear. The meaning of those proteins' changes by CIS and Gln supplementation revealed by this study would deserve further studies in relation with etiology of chronic stress-induced depression and development for new antidepressants.

High molecular-weight (~60 kDa) SNAT2 decreased, whereas 50-kDa SNAT2 accumulated in the PFC following CIS. It was previously reported that the 60-kDa band is a fully glycosylated mature cell-surface transporter, while the lighter band is a partially processed immature intracellular SNAT2 [16]. The decrease of mature SNAT2 and increase of immature SNAT2 by CIS could

be due to the blocking or delay of normal protein processing. The other SNATs (SNAT1, SNAT3, and SNAT5) did not show any significant difference in expression between the mature and immature forms (Fig. 2), which means that the protein processing pathway for SNAT2 might have been more sensitive to CIS compared to the other SNATs.

To our knowledge, no studies have examined expressional changes of GLT1 and SNATs relative to plasma CORT levels in the brain. Our results showed that GLT1, SNAT2, and SNAT5 expression correlated with plasma CORT levels (Fig. 4). The relationship between CORT and these transporters should be further studied to clarify the precise regulatory mechanism of GLT1, SNAT2, and SNAT5 expression and their roles in the etiology of depression.

In summary, the results showed that CIS disturbed the Glu-Gln cycle in the PFC. To the best of our knowledge, this is the first study to investigate expressional changes of Glu-Gln cycle proteins as a whole in response to chronic stress. Our findings could help identify new antidepressant targets related to chronic stress. In addition, the results demonstrate that Gln supplementation facilitates normal Glu-Gln cycling under stressful conditions, consistent with our previous studies demonstrating the antidepressant potential of Gln [3, 14]. In conclusion, CIS decreased Glu and Gln transporter levels, and Gln supplementation increased their expression in the PFC, suggesting that Glu and Gln transporters have important roles in depression pathogenesis and the antidepressant effect of Gln. However, to confirm our result that Gln is a potent antidepressant, the effects of Gln supplementation after CIS, but not during CIS, should be evaluated in further studies.

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