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The $GABA_B$ receptor associates with regulators of G-protein signaling 4 protein in the mouse prefrontal cortex and hypothalamus

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Regulators of G-protein signaling (RGS) proteins regulate certain G-protein-coupled receptor (GPCR)-mediated signaling pathways. The $GABA_B$ receptor ($GABA_BR$) is a GPCR that plays a role in the stress response. Previous studies indicate that acute immobilization stress (AIS) decreases RGS4 in the prefrontal cortex (PFC) and hypothalamus (HY) and suggest the possibility of a signal complex composed of RGS4 and $GABA_BR$. Therefore, in the present study, we tested whether RGS4 associates with $GABA_BR$ in these brain regions. We found the co-localization of RGS4 and $GABA_BR$ subtypes in the PFC and HY using double immunohistochemistry and confirmed a direct association between $GABA_{B2}R$ and RGS4 proteins using co-immunoprecipitation. Furthermore, we found that AIS decreased the amount of RGS4 bound to $GABA_{B2}R$ and the number of double-positive cells. These results indicate that $GABA_BR$ forms a signal complex with RGS4 and suggests that RGS4 is a regulator of $GABA_BR$. [BMB Reports 2014; 47(6): 324-329]

INTRODUCTION

It is well known that regulators of G-protein signaling (RGS) proteins negatively regulate G-protein-coupled receptor (GPCR) signaling pathways via the GTPase-activating property of the RGS domain (1). During stress response, numerous GPCR-mediated signaling pathways are activated or inactivated in the brain (2, 3). We previously investigated acute stress-responsive RGS proteins using a 2-h acute immobilization stress (AIS) paradigm and found a reduction of RGS4 in the prefrontal cortex (PFC) and hypothalamus (HY) of mice (4). RGS4 mRNA is relatively abundant in several brain regions within the stress response circuitry, such as the cerebral cortex, amygdala, thalamus, paraventricular nucleus (PVN) of the HY, and locus coeruleus (LC) (5), and RGS4 mRNA levels in the PVN and LC are regulated in opposite directions by chronic unpredictable stress and corticosterone (6). RGS4 regulates the signaling of several $G\alpha_{i/o}$- and $G\alpha_q$-coupled receptors including group I metabotropic glutamate receptors (mGluRs) (7), μ-opioid receptors (8), $M_{1\text{-}4}$ muscarinic receptors (9), 5-hydroxytryptamine$_{1A/2A}$ (serotonin) receptors (10), and α2A-adrenergic receptors (11). Moreover, some RGS4-related GPCRs are thought to be activated during acute stress, when RGS4 is reduced (4).

To terminate acute stress responses, the inhibition of corticotrophin releasing hormone (CRH) is critical (12) and is mainly regulated by GABA-ergic signaling (13-15). Among GABARs, $GABA_AR$ and $GABA_CR$ are ionotropic receptors, and $GABA_BR$ is a GPCR (16). $GABA_BR$ is composed of $GABA_{B1}R$ and $GABA_{B2}R$ subunits, with $GABA_{B1}R$ containing the GABA binding site and $GABA_{B2}R$ responsible for $G\alpha_{i/o}$ protein activation (17). $GABA_BR$ is involved in stress-related physiological responses, including modulation of plasma interleukin-6 (IL-6) levels (18), antinociception (19), and mood disorders (20). Moreover, a recent fluorescence resonance energy transfer (FRET) study suggests that the $GABA_BR$ signaling complex interacts with RGS4 (21). In the present study, therefore, we examined whether RGS4 proteins associate with $GABA_BR$ in the mouse brain upon AIS.

RESULTS

AIS induced opposite changes in levels of RGS4 and plasma corticosterone

We confirmed the specificity of anti-RGS4 primary antibody using preabsorption with blocking peptide (Supplementary Fig. 1). The 2-h AIS paradigm is known to decrease RGS4 in the brain (4), and acute stress is known to increase corticosterone

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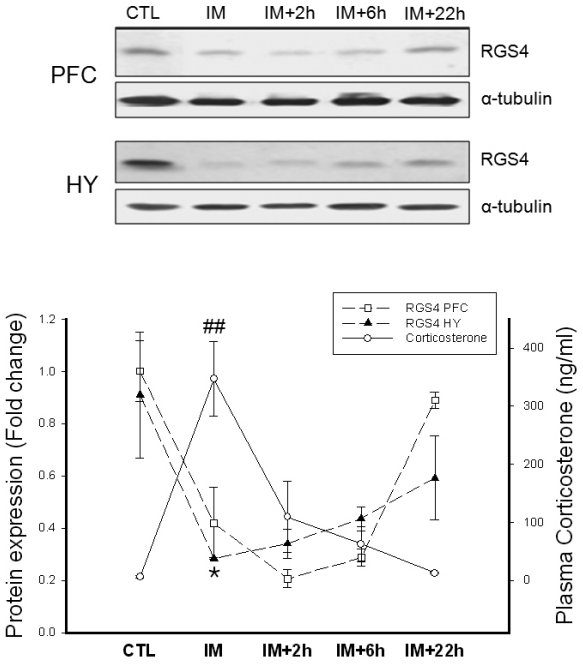

Fig. 1. RGS4 protein immediately decreased after acute stress in the PFC ($F_{(4,9)}$ = 15.43, P < 0.001) and HY ($F_{(4,9)}$ = 3.892, P = 0.0420) but returned to control levels 22 h after termination of IM. CTL, control IM, 2-h immobilization stress IM+2h, 2 h after termination of IM IM+6 h, 6 h after termination of IM IM+22h, 22 h after termination of IM. *P < 0.05, Dunnett *post-hoc* test. Plasma corticosterone levels quickly increased after IM and returned to basal levels 22 h after termination of IM ($F_{(4,25)}$ = 5.693, P = 0.002). $^{\#\#}$P < 0.01, Dunnett *post-hoc* test.

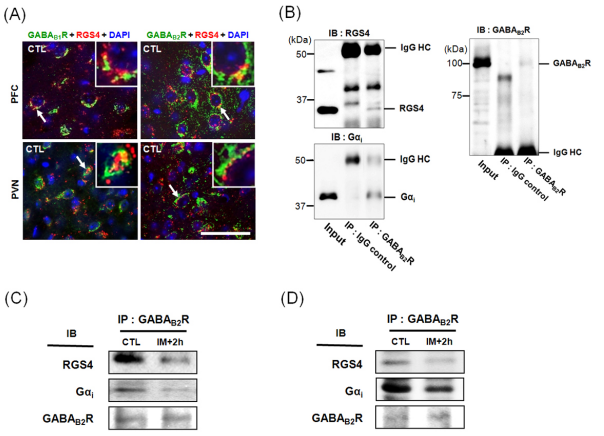

Fig. 2. (A) RGS4 (red) and GABA$_B$R (green) were co-localized (yellow) in the PFC and PVN region of the HY in the CTL group. Each arrow indicates the area of magnification (insets). Scale bar = 50 μm. (B) RGS4 and Gα_i co-precipitated with GABA$_{B2}$R. Protein was immunoprecipitated with anti-GABA$_{B2}$R antibody or normal rabbit serum (IgG control) and immunoblotted (IB) by anti-RGS4, anti-Gα_i, or anti-GABA$_{B2}$R antibody. Input protein (10 μg) was loaded in parallel. There were no size-matched bands in the IgG control lanes, indicating the specificity of co-IP with anti-GABA$_{B2}$R antibody. Co-IP of the GABA$_B$R signal complex with anti-GABA$_{B2}$R-antibodies from the PFC (C) and HY (D) in CTL and IM+2h groups. The precipitate was IB with the indicated antibodies. Note that the amount of RGS4 and Gα_i bound to GABA$_{B2}$R were decreased in the IM+2h group. Data are representative of results from at least three independent experiments (n = 3-5 per group). Similar results were obtained from each experiment.

(22). A previous study also reports that RGS4 mRNA expression in the PVN is decreased by corticosterone (6). Therefore, we investigated temporal changes of RGS4 and plasma corticosterone levels after AIS. RGS4 protein was reduced in the PFC and HY immediately after 2 h immobilization (IM) (Fig. 1). RGS4 protein expression levels remained reduced 6 h after termination of IM (IM+6h) but recovered to control levels after 22 h (IM+22h). The lowest levelof RGS4 protein in the PFC and HYafter AIS was approximately 30% of control levels. In contrast to RGS4, plasma corticosterone levels rapidly increased after IM but declined 6 h after termination of IM. Corticosterone levels returned to basal levels 22 h after termination of AIS (IM+22h).

RGS4 and GABA$_B$R were co-localized and co-precipitated in the PFC and HY

To identify the positional relationship of RGS4 and GABA$_B$R in the PFC and HY, double-immunohistochemistry (D-IHC) was performed using antibodies for RGS4 and GABA$_{B1}$R or GABA$_{B2}$R subunits (Fig. 2A). Double-positive cells were found in the PFC and PVN region of the HY. However, not all RGS4 signals overlapped with GABA$_{B1}$R or GABA$_{B2}$R signals, suggesting that RGS4 also modulates other GPCRs such as α_{2A}-adrenergic receptors or mGluR5 (11, 23).

To assess whether the endogenous RGS4 protein physically interacts with GABA$_B$R *in vivo*, co-immunoprecipitation (IP) was performed under non-denaturing conditions (Fig. 2B). RGS4 and G$_{\alpha i}$ were detected in the anti-GABA$_{B2}$R antibody precipitates, indicating a direct association between RGS4 and the GABA$_B$R signal complex. A control experiment with normal IgG did not produce size-matched bands for RGS4, G$_{\alpha i}$, or GABA$_{B2}$R of the total input protein. After establishing the procedure for co-IP of anti-GABA$_{B2}$R antibody, we investigated whether the amount of RGS4 bound to the GABA$_B$R signal complex was decreased by AIS in the PFC and HY (Fig. 2C and D). We found that precipitates from the anti-GABA$_{B2}$R antibody contained reduced amounts of RGS4 and Gα_i protein from both brain regions in the stressed group (IM+2h) compared with the control (CTL) group.

AIS decreased RGS4/GABA$_B$R double-positive cells

After confirming the association of RGS4 and GABA$_B$R, we investigated whether AIS decreased the number of RGS4/GABA$_{B2}$R double-positive cells. D-IHC was performed on brain tissue from CTL and IM+2h groups (Fig. 3). In the cingulate cortex (Cg1) of the PFC, there were no significant differ-

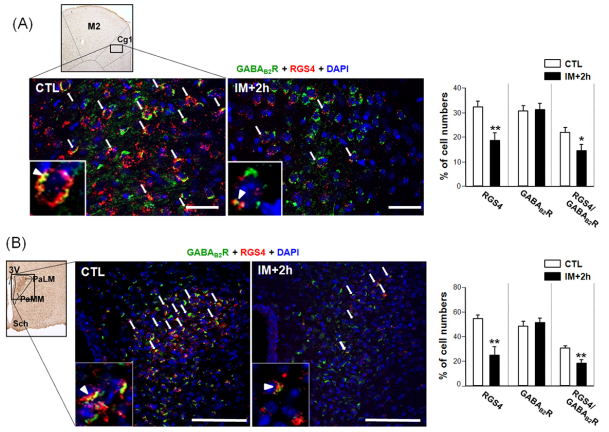

Fig. 3. (A) Expression of RGS4 protein (red) but not GABA$_B$R (green) decreased in the Cg1 region of the PFC in the IM+2 h group (left panel). Arrows indicate co-localization (yellow) of RGS4 and GABA$_{B2}$R. Scale bars = 50 μm. The percentage of RGS4-positive and RGS4/GABA$_{B2}$R double-positive cells was significantly decreased in the IM+2h group (n = 4, *P < 0.05). (B) Expression of RGS4 protein but not GABA$_B$R decreased in the lateral and medial magnocellular part (PaLM, PaMM) of the PVN region of the HY in the IM+2 h group (left panel). Arrows indicate co-localization of RGS4 and GABA$_{B2}$R. Scale bars = 100 μm. The percentage of RGS4-positive and RGS4/GABA$_{B2}$R double-positive cells was significantly decreased in the IM+2h group (n = 4, **P < 0.01).

ences between groups in the number of GABA$_{B2}$R-positive cells, but the number of RGS4-positive cells and RGS4/GABA$_{B2}$R double-positive cells was lower in the IM+2h group compared with the CTL group (Fig. 3A and Supplementary Fig. 2). In the PVN, AIS decreased the number of RGS4-positive cells, resulting in a lower number of RGS4/GABA$_{B2}$R double-positive cells in the IM+2h group compared with the CTL group (Fig. 3B and Supplementary Fig. 3).

DISCUSSION

We found that the AIS-responsive RGS4 protein was associated with GABA$_B$R in the PFC and HY, suggesting that some stress-related GABAergic phenomena may be enhanced in regions, when RGS4 is decreased. In the Cg1 of the PFC, AIS decreased the number of RGS4 and GABA$_B$R double-positive cells (Fig. 3A). It is not clear, however, which functions of the Cg1 might be affected by AIS-induced changes in the RGS4-GABA$_B$R complex, as the Cg1 takes part in a variety of neural processes including attention (24), emotion (25), and cognition (26). Although normal and disordered functions of the Cg1 are modulated by stressful environments (27), many GPCRs are involved in these processes, including $M_{2\text{-}3}$muscarinic, μ-opioid, α2-adrenergic, 5-hydroxytryptamine1A, and GABA$_B$R (28). Interestingly, with the exception of GABA$_B$R, these receptors have already been recognized as RGS4-related GPCRs (8-11), and GABA$_B$R was found to be an RGS4-bound GPCR in the present study (Fig. 2). Therefore, it is possible that additional RGS4-related GPCR signaling pathways may be changed by AIS. Also, stress-induced changes in neural activity could be accomplished by the cooperation of several GPCRs and not modulated by a single GPCR signaling pathway. This possibility is supported by our D-IHC results, which show partial co-localization of RGS4 with GABA$_B$R (Fig. 2A and 3A).

In the PVN of the CTL group, RGS4 and GABA$_B$R double-positive cells were mainly localized in the parvocellular region, which contains CRH neurons (29). The acute stress response begins with the release of CRH and ends with the blockade of CRH release from CRH-containing neurons via glucocorticoid negative feedback (6, 22). CRH release is negatively regulated by GABA in the PVN, which is largely mediated by GABA$_B$R (15, 30). In the present study, we demonstrated a direct association of RGS4, GABA$_{B2}$R, and Gα$_i$ through *in vivo* co-IP (Fig. 2B) and showed that AIS decreased the amount of RGS4 protein bound to the GABA$_B$R complex in the HY (Fig. 2D), consistent with a recent FRET study (21). Additionally, the number of RGS4 and GABA$_B$R double-positive cells was significantly decreased in the medial parvocellular region of PVN (Fig. 3B), where nearly half of the cells have been identified as GABAergic (31). Thus, the decrease in RGS4 bound to GABA$_B$R may result in a decrease in CRH release, which is supported by the observation of opposite changes in corticosterone and RGS4 levels after AIS (Fig. 1). However, other RGS4-related GPCRs also exist in the PVN, and it has recently been suggested that the decrease in RGS4 by acute stress may lead to an increase in mGluR and μ-opioid receptor signaling in the PVN, which may contribute to stress adaptation (32). Therefore, cooperative signaling changes related to RGS4 may also occur in the PVN during acute stress.

Although we cannot conclusively remark on its physiological significance, we confirmed the association between RGS4 and GABA$_B$R in the PFC and HY after acute stress using *in vivo* co-IP and D-IHC. This association suggests a putative regulatory mechanism of GABA$_B$R signaling in these two brain regions during acute stress responses. In previous studies, RGS2 and RGS7 were found to modulate G-protein-gated inwardly rectifying K^+ channels coupled with GABA$_B$R in the ventral tegmental area and hippocampus, respectively (33, 34). A modulatory role of RGS6 in GABA$_B$R signaling was also reported in the cerebellum (35). These findings suggest that the modulation of various GABA$_B$R signaling pathways is achieved by regionally and functionally specific RGS proteins. Therefore, future studies should aim to elucidate the role of RGS4 in the regulation of GABA$_B$R signaling in the PFC and HY.

MATERIALS AND METHODS

Animals and AIS treatment

Nine-week-old male C57BL/6 mice (SPF grade, Hana, Co. Ltd., Korea) were housed in a temperature-controlled (22°C) envi-

ronment under a 12 h light/dark cycle (lights on at 6 : 00 AM), with free access to laboratory chow and water. The animals were habituated for 1 week before experiments. Mice in stress groups were placed in a plastic restrainer for 2 h in a separate room equipped with a 200-lux light and maintained at 22°C. Mice in the CTL group were kept in their home cage before sacrifice. Groups were divided into CTL, 2-h IM, 2 h after the termination of IM (IM+2h), 6 h after the termination of IM (IM+6h), and 22 h after the termination of IM (IM+22h). All procedures were approved by the Gyeongsang National University Institution Animal Care & Use Committee (GLA-100917-M0093).

Enzyme-link immunosorbent assay (EIA) of plasma corticosterone

Mouse blood was collected in vacutainers containing K3EDTA. Plasma was isolated via centrifugation at 1,000×g for 15 min at 4°C. The samples were stored at −80°C until the assay was performed. Quantification of plasma corticosterone levels was carried out using the corticosterone EIA kit (Cayman, MI, USA) according to the manufacturer's protocol.

Western blot analysis

Western blot analysis was performed as previously described (4). Briefly, protein-transferred membranes were blocked and incubated with primary antibodies (anti-RGS4, 1 : 500, SC-6204; anti-$GABA_{B2}R$, 1 : 100, SC-28792; anti-$G_{\alpha i\text{-}3}$, 1 : 500, SC-262; Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were detected with an enhanced chemiluminescence detection kit (Amersham Biosciences, Munich, Germany) according to the manufacturer's protocol. For quantification of the results, each band density was read by SigmaGel software (Sigma). Each density was normalized using the corresponding α-tubulin density as an internal control.

Immunohistochemistry

For histological studies, mice were perfused, and brain tissues were immunostained as previously described with some modifications (36). For D-IHC, sections were incubated with mixed primary antibody solutions containing RGS4 (1 : 200) + $GABA_{B1}R$ (1 : 100, SC-14006, Santa Cruz Biotechnology, Santa Cruz, CA) or RGS4 (1 : 200) + $GABA_{B2}R$ (1 : 100) at 4°C overnight. To detect primary antibodies, Alexa Fluor (AF)-594- and AF-488-conjugated secondary antibodies were used. Sections were mounted on gelatin-coated slides and coverslipped using a wet mount solution (Invitrogen, Carlsbad, CA). To count immuno-positive cells, four images (0.015 mm^2) were obtained from each group. Single- and double-positive cell numbers for RGS4 and $GABA_{B2}R$ and total cell number covering the entire area were separately counted for each image (37). Images were obtained using a spinning disk confocal microscope equipped with an Olympus Disk Spinning Unit (BX2-DSU) (38).

Co-immunoprecipitation

Total protein extracts were prepared in RIPA buffer (Tris-HCl: 50 mM, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) by grinding with a disposable polypropylene grinder followed by ultra-sonication. Protein concentration was assayed using the bicinchoninic acid assay (BCA) method. Lysates were stored at −70°C in aliquots before further use. Equal amounts of protein lysates (800 μg each) were precleared once with 50 μl TrueBlot anti-rabbit IgG IP beads (eBioscience, San Diego, CA) on ice for 30 min and centrifuged at 10,000×*g* for 3 min. The supernatant was incubated with 5 μg primary antibody on ice for 1 h. After further incubation with 50 μg of anti-rabbit IgG beads for 2 h, immune complexes were collected by centrifugation at 10,000×*g* and washed three times using 500 μl RIPA buffer. Immunoprecipitates were mixed with sample buffer, dissociated by heating for 10 min, resolved with SDS-PAGE, and analyzed by western blotting (39).

Statistical analyses

Data were analyzed using one-way analysis of variance (ANOVA) and Dunnett *post-hoc* tests. For comparisons between two groups, *t* tests were used (GraphPad Prism 5.01). Data are presented as mean ± standard error (SE). Statistical significance was set at $P < 0.05$.

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