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Acute Stress Responsive RGS Proteins in the Mouse Brain

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Regulator of G-protein signaling (RGS) proteins play an important role in G-protein coupled receptor (GPCR) signaling and the activity of some GPCRs is modulated via RGS protein levels during stress response. The aim of this study was to investigate changes in RGS protein mRNA expressions in the mouse brain after 2h restraint stress. The mRNA level of 19 RGS proteins was analyzed using real-time PCR in six brain regions, which included the prefrontal cortex, amygdala, hippocampus, hypothalamus, striatum, and pituitary gland, from control and stressed mouse. We found that the level of mRNA of each RGS varied according to brain region and that two to eight RGS proteins exhibited changes in mRNA levels in each brain region by restraint stress. It was also revealed that RGS4 protein amount was consistent with mRNA level, indicating RGS4 protein may have regulatory roles in the acute stress response.

INTRODUCTION

G-protein coupled receptors (GPCRs) are major components of signal organization and propagation in the central nervous system (CNS) of higher organisms. There is a vast body of evidence regarding the regulatory mechanisms of GPCR-mediated signal transduction. The trans-synaptic activation of GPCRs is considered as a main pathway of basic neural functions and behaviors; thus, numerous drugs have been developed to block or enhance the activity of specific GPCRs (Cristalli et al., 2008; Rodriguez-Puertas and Barreda-Gomez, 2006).

The stress response is initiated through the hypothalamus-pituitary-adrenal (HPA) axis leading an increase in serum corticosteroid levels (Dedovic et al., 2009). In addition to the effect of corticosteroids, other mechanisms are turned on to terminate the acute stress response and promote adaption to this stress (e.g., negative feedback). It has been being suggested that, during these processes, numerous GPCR-mediated signaling pathways are activated or inactivated in the brain to respond to, and overcome, stressful circumstances (Kiss and Aguilera, 2000).

Regulator of G-protein signaling (RGS) proteins, which were identified in 1996 and act to regulate negatively the signaling mediated by GPCRs (Dohlman et al., 1996), are considered as new targets to control the GPCR signal-transduction process (Chasse and Dohlman, 2003). The measurement of the mRNA levels of RGS proteins provides insights into their tissue specificity and regulation (Kurrasch et al., 2004). As RGS proteins are involved in the control of the frequency and duration of GPCR signaling, the information on the distribution and regulation of RGS proteins will likely contribute to the elucidation of the mechanisms that underlie the related GPCR signaling pathways. Moreover, as many studies indicate that the regulation of RGS protein expression is mediated by physiological stimuli transduced by G proteins (Ding et al., 2006; Dohlman et al., 1996; Saugstad et al., 1998), it may be possible to monitor the activity of GPCR signaling cascades by measuring changes in the levels of expression of RGS proteins in specific cells or tissues.

Therefore, the present study aimed to identify acute restraint stress responsive RGS proteins using a 2 h restraint stress and real-time PCR analysis in the mouse brain. We found that the repertoire of changes in RGS protein mRNA expression levels in each brain region tested had a region-specificity.

MATERIALS AND METHODS

Animals and restratint stress treatment

Male nine-week-old C57BL/6 mice (SPF grade, Hana, Co. Ltd., Korea) were housed in a temperature-controlled (22°C) environment under a 12 h light/dark cycle (lights on at 6 AM), with free access to laboratory chow and water. The animals were habituated for one week before experiments. Animals in the stress group were subjected to plastic restrainer for 2 h in a separate room equipped with a 200 lux light and maintained at 22°C temperature. Control mice were kept in their home cage before being sacrificed. Mice were treated in accordance with the standard guidelines for laboratory animal care at the animal facility of the Gyeongsang National University School of Medicine.

Total RNA isolation and real-time PCR (RT-PCR)

Mice were anesthetized with CO_2 and decapitated. The brains were removed and dissected carefully into six discrete regions

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Table 1. mRNA expression amount (A.U.) of 19 RGS proteins in six brain regions

	Prefrontal cortex	Hippocampus	Amygdala	Striatum	Hypothalamus	Pituitary gland
RGS1	1.3 ± 0.2	1.2 ± 0.4	1.2 ± 0.3	1.4 ± 0.2	0.9 ± 0.3	9.0 ± 1.5
RGS2	855.8 ± 12.3	1141.5 ± 245.6	1278.8 ± 36.0	3523.1 ± 94.1[a]	864.7 ± 260.5	7306.5 ± 37.8[a]
RGS3	35.0 ± 3.8	3.2 ± 0.8	40.3 ± 2.9	12.0 ± 0.3	48.7 ± 6.2	112.1 ± 50.1
RGS4	1645.4 ± 40.0[a]	111.7 ± 2.6	492.3 ± 34.6	1845.0 ± 231.7	499.0 ± 123.3	376.5 ± 155.5
RGS5	284.4 ± 15.9	135.5 ± 11.5	164.1 ± 8.5	208.0 ± 24.3	234.0 ± 41.6	791.7 ± 255.4
RGS6	44.9 ± 2.8	5.4 ± 1.1	14.1 ± 0.3	15.5 ± 4.2	18.5 ± 4.1	1.4 ± 0.5
RGS7	390.4 ± 7.9	228.6 ± 11.6	261.7 ± 12.7	240.5 ± 31.7	318.4 ± 46.0	335.0 ± 75.2
RGS8	169.8 ± 12.9	310.5 ± 60.0	89.7 ± 8.7	604.0 ± 108.1	203.1 ± 60.6	64.7 ± 18.8
RGS9	26.1 ± 1.0	33.0 ± 2.1	42.3 ± 11.6	1961.7 ± 372.8	99.4 ± 27.9	235.0 ± 70.1
RGS10	762.1 ± 25.6	3303.0 ± 971.5[a]	1180.7 ± 55.5	2339.7 ± 338.0	3303.0 ± 971.5[a]	388.7 ± 24.0
RGS11	67.7 ± 3.7	47.8 ± 11.7	25.0 ± 6.1	65.7 ± 14.2	47.8 ± 11.7	38.0 ± 27.0
RGS12	288.0 ± 14.8	300.0 ± 81.6	402.0 ± 25.1	353.0 ± 52.3	300.0 ± 81.6	261.3 ± 94.8
RGS13	6.1 ± 3.7	16.9 ± 3.6	1.9 ± 0.3	4.1 ± 2.4	16.9 ± 3.6	14.0 ± 2.3
RGS14	74.5 ± 6.7	47.6 ± 10.1	120.7 ± 16.8	120.3 ± 10.3	47.6 ± 10.1	1.0 ± 0.1
RGS16	11.9 ± 2.2	15.9 ± 1.5	10.0 ± 1.3	5.6 ± 1.3	15.9 ± 1.5	99.0 ± 11.5
RGS17	1037.4 ± 29.0	1984.0 ± 597.0	1453.0 ± 157.7[a]	1205.3 ± 173.5	1984.0 ± 597.0	408.3 ± 32.6
RGS18	12.0 ± 5.2	21.6 ± 3.0	3.5 ± 1.2	4.8 ± 2.4	21.6 ± 3.0	19.7 ± 2.5
RGS19	15.3 ± 1.2	14.0 ± 2.5	11.1 ± 2.4	3.8 ± 0.7	14.0 ± 2.5	14.5 ± 0.7
RGS20	554.0 ± 23.4	688.7 ± 216.1	412.3 ± 33.8	889.3 ± 29.2	688.7 ± 216.1	64.3 ± 9.4

Arbitrary units (A.U.) were calculated through normalization of each RGS mRNA expression value by corresponding GAPDH expression value. Data presented were mean ± SEM (n = 5).
The most abundant RGS protein in each region are indicated by "a".

(prefrontal cortex, striatum, hippocampus, amygdala, hypothalamus, and pituitary gland), which were frozen in liquid nitrogen. The frozen tissues were kept at -80°C until use. Total RNA for RT-PCR analyses was extracted using the Trizol reagent (Sun et al., 2009) and was further purified using an RNeasy kit (Qiagen Inc., USA).

The overall RT-PCR analysis was preformed as described previously (Joo et al., 2009). The first cDNA strand was synthesized from 1 μg of total RNA using MMLV-reverse transcriptase (Promega) and $(dT)_{15}$ primers. The primers used to detect RGS protein mRNAs were same as described by Kurrasch et al. (Kurrasch et al., 2004). RT-PCR amplification and relative quantification were achieved using a LightCycler 480 (Roche). All templates were amplified using the following protocol: the polymerase was activated and cDNA was denatured via a preincubation of 10 min at 95°C; the template was amplified for 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 5 s, and extension at 72°C for 15 s. Advanced relative quantification was performed using the ratio of target (RGS) to reference (GAPDH) Cp values, which was calculated automatically using the LC480 software (version 4.0, Roche).

Western blot analysis

Western blot analysis was performed as previously described (Kim et al., 2006). Briefly, total protein extracts were prepared in tri-detergent lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 0.5 μg/ml leupeptin) by grinding with disposable polypropylene grinder followed by ultra-sonication. Protein samples (20 μg each) were separated by SDS-PAGE and transferred to nitrocellulose paper. The nitrocellulose membrane was blocked with 1% bovine serum albumin and 5% skim milk, and incubated with anti-RGS4 antibody (1:500, SC-6204, Santa Cruz Biotechnology, USA). Bound antibodies were detected with an enhanced chemiluminescence detection kit (Amersham Biosciences, Germany) according to the supplied protocol. For quantification of the result, each band density was read by SigmaGel software (Sigma, USA). Each density was normalized using each corresponding α-tubulin density as an internal control.

Data manipulation and statistical analyses

The data presented as mRNA expression level were the normalized relative quantitative values by each corresponding GAPDH expression level as an internal control. The fold changes were used to compare the mRNA and protein expression levels of individual RGS proteins between the control and stressed groups after statistical evaluation, which was performed using a *t* test (SigmaStat Ver. 3.5). Significance of results was set at $P < 0.05$.

RESULTS AND DISCUSSION

The present study represents acute restraint stress responsive RGS proteins for the first time. It also supports several important previous findings including the existence of brain-enriched RGS proteins (Kurrasch et al., 2004; Larminie et al., 2004) and the differential expression of RGS protein mRNAs in different brain regions (Gold et al., 1997).

It is well known that six selected regions investigated in the present study are involved in behavioral and physiological responses to stressful circumstances and are affected by corticosteroids (Dedovic et al., 2009). As expected, the expression amount of each RGS protein mRNA was region specific. In the prefrontal cortex, RGS4 was the most abundant RGS protein. In other brain regions, however, other RGS proteins were more abundant than RGS4, i.e., RGS2 was more abundant in the

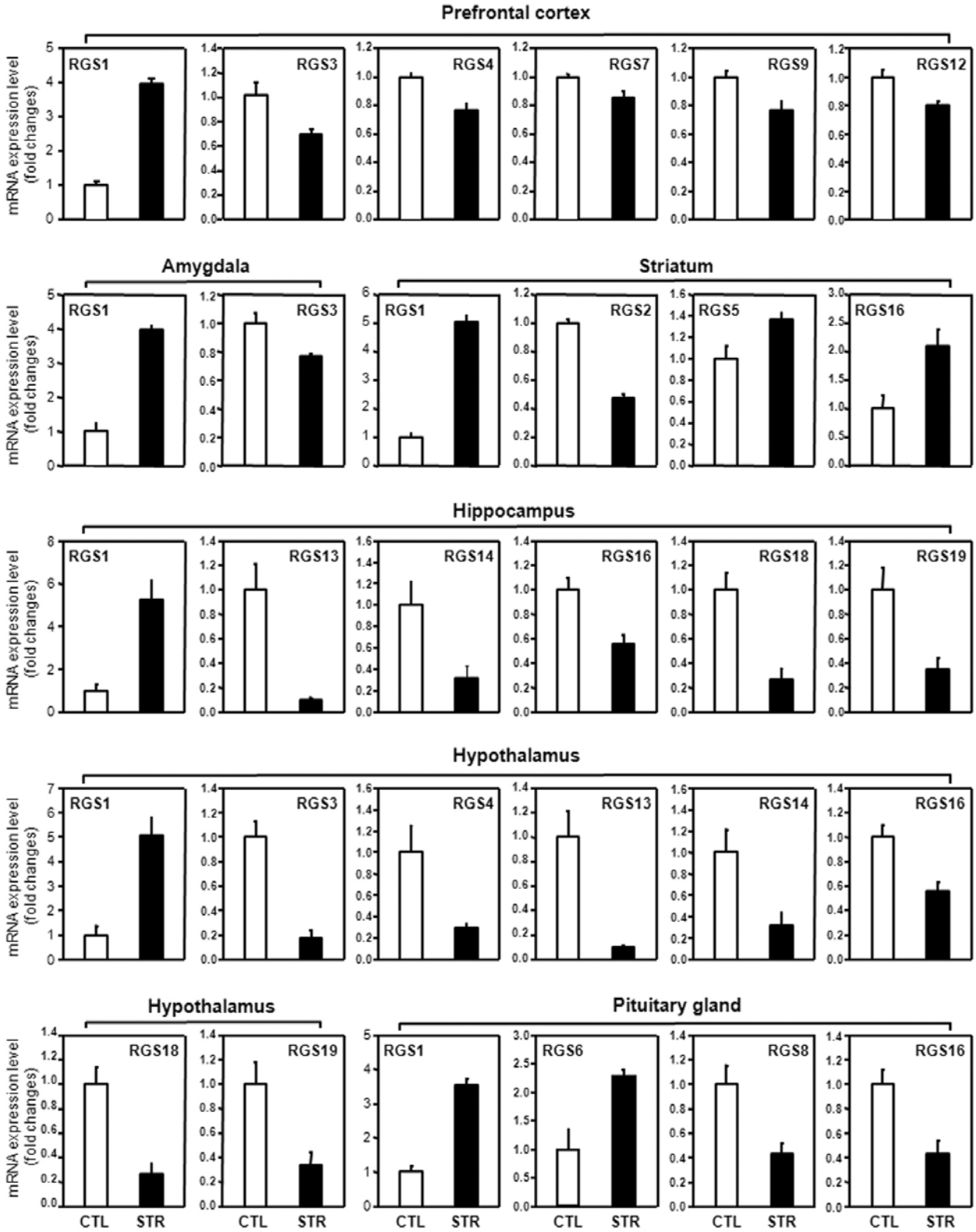

Fig. 1. Acute restraint stress responsive RGS proteins in six regions of the mouse brain. The expression values of the 19 RGS proteins from the control (CTL) and stress (STR) groups were statistically evaluated using a *t*-test. RGS proteins of which mRNA expression levels were significantly different between the CTL and STR groups ($P < 0.05$) were selected. The repertoire of restraint stress responsive RGS proteins was region specific, which implies the presence of region-specific responsiveness in the mouse brain against acute stress. Data presented are the mean + SE (n = 5).

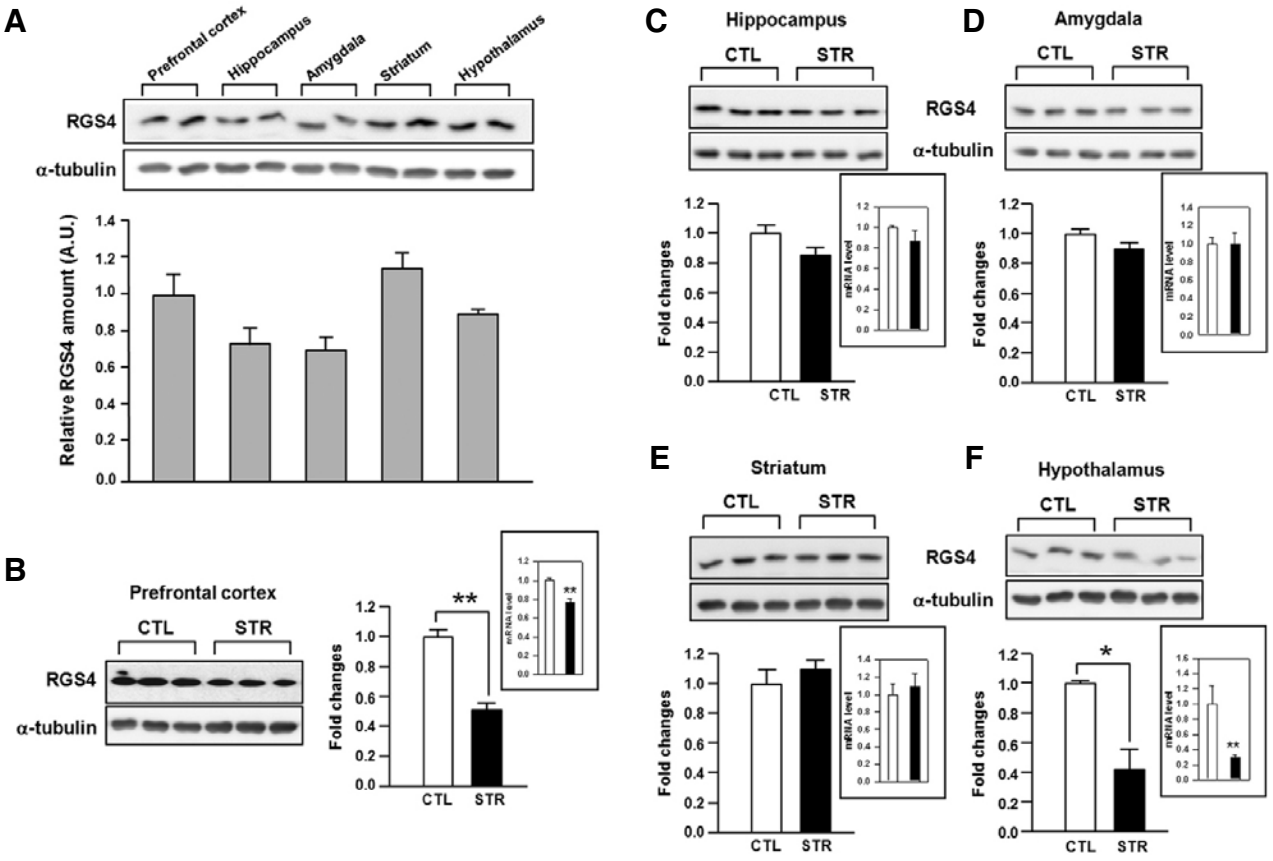

Fig. 2. Western blot analysis was preformed to investigate the regional expression level of RGS4 protein in the five brain regions from control mouse (A). The regional protein expression level of RGS4 was consistent with mRNA expression level. The change of RGS4 protein expression was also tested using 2 h restraint stressed mouse (STR) and control mouse (CTL) in the prefrontal cortex (B), hippocampus (C), amygdala (D), striatum (E), and hypothalamus (F). The graphs in boxes represent corresponding RGS4 mRNA changes by acute restraint stress. Graphs in (B) and (F) are the same used in Fig. 1 and others were made by same method for Fig. 1. The RGS4 protein was decreased in the prefrontal cortex and hypothalamus by acute restraint stress. $^{*}P < 0.05$, $^{**}P < 0.01$, Data = mean + SE (n = 3).

striatum and pituitary gland, and RGS10 was predominant in the hippocampus and hypothalamus. In the amygdala, the mean expression levels of RGS2, RGS10, and RGS17 were similar and more than RGS4 (Table 1). However, the patterns of lower expression levels of the RGS proteins were similar in the five brain regions except for pituitary gland. The RGS1, 3, 6, 9, 11, 13, 16, 18, and 19 were expressed at the low levels in those regions. It was interesting to note that the pituitary gland exhibited a level of expression of the RGS2 that was significantly higher compared with other brain regions and with other RGS proteins within the pituitary gland, which suggests specific roles for RGS2 in this structure (Wilson et al., 2005).

Figure 1 shows the RGS proteins that exhibited a significant change by the 2 h restraint stress. Although all the RGS proteins shown in Table 1 were investigated, only a few of them showed acute restraint stress responsive changes. Moreover, this repertoire seemed to be region specific, with the exception of the RGS1, which was increased in all six regions tested. In addition to RGS1, seven RGS proteins in the hypothalamus, five in the prefrontal cortex and hippocampus, three in the striatum and pituitary gland, and one in the amygdala exhibited significant differences in their mRNA expression levels by restraint stress. RGS3, 4, 7, 9, and 12 were decreased in the prefrontal cortex. Only RGS3 was downregulated in the amygdala. In the striatum, RGS5 and 16 were upregulated, whereas RGS2 was downregulated. RGS13, 14, 16, 18, and 19 were decreased in the hippocampus and RGS3, 4, 13, 14, 16, 18, and 19 were downregulated in the hypothalamus. Finally, the expression of RGS6 was increased and the expression of RGS8 and 16 was decreased in the pituitary gland (Fig. 1).

Regarding the nature of RGS protein, the restraint stress responsive RGS proteins may play specific roles in acute stress response via the regulation of certain GPCR signaling pathways in specific brain regions (Abramow-Newerly et al., 2006; Ross and Gilman, 1980). However, these supposed roles would likely be independent from the abundance, as both high- and low-expressed mRNA levels of RGS proteins were altered by 2h restraint stress. For example, RGS4, which was the most abundant one, was downregulated in the prefrontal cortex, whereas RGS1, which showed the least abundance, was upregulated in this region (Table 1 and Fig. 1).

As RGS4 is one of the best studied RGS proteins at the biochemical and cellular levels, the protein expression of RGS4 was further investigated five brain regions (Fig. 2). The normal expression level of RGS4 protein in those regions was consistent with the mRNA expression pattern (Table 1 and Fig. 2A). Upon acute restraint stress, RGS4 mRNA was significantly decreased only in the prefrontal cortex and hypothalamus but other regions tested did not show significant changes (Fig. 2). Not only the decrements of RGS4 protein in the prefrontal cor-

tex and hypothalamus were consistent with RGS4 mRNA expression pattern, but the unchanged RGS4 protein expressions in other regions reflected well its own mRNA expression pattern (Fig. 2). These results implicated that RGS4 mRNA transcription would be a main process to regulate RGS4 protein amount in the brain tissue. RGS4 regulates the signaling of several $G\alpha_i$- and $G\alpha_q$-coupled receptors, including group I metabotropic glutamate receptors (Saugstad et al., 1998), μ-opioid receptors (Georgoussi et al., 2006), and M_{1-4} muscarinic receptors (Ding et al., 2006). Because RGS proteins regulate GPCR signaling negatively, the decrement of the RGS4 expression is thought to be closely related to the activation of specific signals by cognate receptors during and/or after restraint stress (Abramow-Newerly et al., 2006). Therefore, it will be warranty further elucidation which specific GPCR-related signaling would be positively regulated by reduced RGS4 protein in both regions.

Even the stress-induced psychiatric illnesses are mainly caused by chronic or by severe-single stressful experiences (Joo et al., 2009; Ponniah and Hollon, 2009), those may be the result of the accumulation of abnormal responses to acute stress. Therefore, it is crucial to determine how the brain acts to overcome or accommodate an acute stress, which may be mediated by GPCR signalings. As there are less numbers of RGS proteins than GPCRs, it would be an easier way to use RGS proteins for the elucidation of the mechanisms that underlie this type of illnesses, as well as for the identification of drug targets. From this perspective, the restraint stress responsive RGS proteins identified in this study may be excellent targets for future investigations of brain responsiveness to stress.

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