Regulator of G-protein signaling 6 (RGS6) promotes anxiety and depression by attenuating serotonin-mediated activation of the 5-HT\textsubscript{1A} receptor-adenyl cyclase axis

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ABSTRACT Targeting serotonin (5-HT) bioavailability with selective 5-HT reuptake inhibitors (SSRIs) remains the most widely used treatment for mood disorders. However, their limited efficacy, delayed onset of action, and side effects restrict their clinical utility. Endogenous regulator of G-protein signaling (RGS) proteins have been implicated as key inhibitors of 5-HT\textsubscript{1A}-Rs, whose activation is believed to underlie the beneficial effects of SSRIs, but the identity of the specific RGS proteins involved remains unknown. We identify RGS6 as the critical negative regulator of 5-HT\textsubscript{1A}-R-dependent antidepressant actions. RGS6 is enriched in hippocampal and cortical neurons, 5-HT\textsubscript{1A}-R-expressing cells implicated in mood disorders. RGS6\textsuperscript{-/-} mice exhibit spontaneous anxiolytic and antidepressant behavior rapidly and completely reversibly by 5-HT\textsubscript{1A}-R blockade. Effects of the SSRI fluvoxamine and 5-HT\textsubscript{1A}-R agonist 8-OH-DPAT were also potentiated in RGS6\textsuperscript{-/-} mice. The phenotype of RGS6\textsuperscript{-/-} mice was associated with decreased CREB phosphorylation in the hippocampus and cortex, implicating enhanced Go\textsubscript{i}-dependent adenyl cyclase inhibition as a possible causative factor in the behavior observed in RGS6\textsuperscript{-/-} animals. Our results demonstrate that by inhibiting serotoninergic innervation of the cortical-limbic neuronal circuit, RGS6 exerts powerful anxiogenic and prodepressant actions. These findings indicate that RGS6 inhibition may represent a viable means to treat mood disorders or enhance the efficacy of serotonergic agents.—Stewart, A., Maity, B., Wunsch, A. M., Meng, F., Wu, Q., Wemmie, J. A., Fisher, R. A. Regulator of G-protein signaling 6 (RGS6) promotes anxiety and depression by attenuating serotonin-mediated activation of the 5-HT\textsubscript{1A} receptor-adenyl cyclase axis. FASEB J. 28, 000–000 (2014). www.fasebj.org

Key Words: mood disorders • SSRIs • GPCRs • animal behavior • cAMP

Deficits in serotonergic neurotransmission have been implicated in a number of mood disorders, including pathological anxiety and depression, which represent large, national socioeconomic health burdens. Anxiety and depression often present as comorbid pathologies, and there is some evidence of a causative link between the two diseases. Current therapeutics target presynaptic reuptake symporters to increase serotonin (5-HT) bioavailability and action at postsynaptic sites primarily located in the hippocampus and prefrontal cortex. Selective 5-HT reuptake inhibitors (SSRIs) remain among the most commonly prescribed drugs in the United States. However, limited efficacy, delayed onset of action, and off-target effects limit their clinical utility, underscoring the need to develop more effectual drugs. Furthermore, because 5-HT activates numerous receptors with divergent and often opposing effects on neuronal signaling, efforts to selectively target receptors associated with the benefi-

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cial actions of SSRIs have proven challenging (1), and the exact molecular targets necessary for the anti-anxiety and antidepressant actions of 5-HT remain unclear.

Nearly all 5-HT receptors belong to the G-protein-coupled receptor (GPCR) class of cell surface receptors and are, thus, subject to regulation by regulator of G-protein signaling (RGS) proteins, GTPase-activating proteins (GAPs) for Gα subunits. By stabilizing the transition state in GTP hydrolysis by Gα, RGS proteins accelerate termination of GPCR signaling and limit the extent of the cellular response to GPCR stimulation (2). Mice expressing a knock-in mutation in Gα12α rendering the protein insensitive to RGS protein-mediated regulation exhibit spontaneous anxiolytic and antidepressant behavior due to potentiation of 5-HT receptor 1A (5-HT1A-R) signaling (3). The RGS protein superfamily contains some 30 members, and studies in Gα12(G148S)-knock-in mice have failed to identify the specific RGS proteins responsible for anxiogenic and prodepressant blockade of 5-HT1ARs in vivo.

RGS6 belongs to the R7 subfamily of RGS proteins, characterized by their 3-domain structure. The RGS domain confers functional GAP activity directed toward Gα12/13 and 2 additional domains, the disheveled, EGL-10, pleckstrin homology (DEP) and Gγ-like (GGL) domains, control protein stability, localization, and protein-protein interactions. The GGL domain facilitates interaction between R7 family members and the atypical Gβ subunit Gβ5. Complex formation between R7 family members and Gβ5 is required for the stable expression of both proteins (4). Here we investigate the involvement of RGS6, expressed in hippocampus and cortex, in anxiety and depression behaviors in mice. We recently noted. For all animal experiments, analyses were performed by an observer blinded to mouse genotype and drug treatment. All animal experiments were performed in agreement with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Materials and Methods**

**Materials**

WAY-100635, 8-hydroxy-2-(di-N-propylamino)tetratin (8-OH-DPAT), fluvoxamine, forskolin, and β-actin antibody were obtained from Sigma (St. Louis, MO, USA). SCH-50911 was from R&D Systems (Minneapolis, MN, USA). We developed the RGS6L and pan-RGS6 antibodies in rabbits. Antibodies to Gβ5 and Gγ7 were generously provided to us by Dr. Jason Chen (Virginia Commonwealth University, Richmond, VA, USA). Antibodies for protein kinase B (Akt), phospho-Akt (S473/T308), phospho-mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3β (GSK3β), phospho-GSK3β(S9), phospho-PKA substrate, cyclic AMP (cAMP)-response element-binding protein (CREB), and phospho-CREB(S133) were from Cell Signaling Technology (Boston, MA, USA). 5-HT1AR antibody and antibody for MAPK were from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies targeting microtubule-associated protein 2 (MAP2) and α-tubulin were from EMD Millipore (Billerica, MA, USA).

**Mice**

We generated RGS6−/− mice as described previously (5). Unless otherwise noted, experiments were performed with age-matched wild-type (WT; RGS6+/+), heterozygous (RGS6+/−), and knockout (RGS6−/−) mice (10–12 wk of age) backcrossed onto a C57BL6 background for 5 generations. Mice were housed on a 12-h light-dark cycle, and experiments were performed during the light cycle. Animals of both sexes were used for behavioral experiments, as we observed no sex-specific differences in mouse performance. Animals naive to each paradigm were used for all behavioral experiments. Drugs were administered 30 min prior to behavioral testing via intraperitoneal (i.p.) injection unless otherwise noted. For all animal experiments, analyses were performed by an observer blinded to mouse genotype and drug treatment. All animal experiments were performed in agreement with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Animal behavioral tests**

Anxiety was assessed in mice placed in an automated 40.6- × 40.6- × 36.8-cm open field (San Diego Instruments; San Diego, CA, USA) for 30 min. Center activity was defined as the number of photobeam breaks occurring in the center area (15.2×15.2 cm) and expressed as a percentage of the total. Rearing was also monitored. The elevated plus maze apparatus has been described previously (6). After placement of mice in the maze center, behavior was documented for 5 min and scored for total time spent in the open arms of the maze and the number of open arm entries. Novelty-induced hypophagic behavior was assessed in nonstarved animals placed in home or novel cage environments, as outlined previously (7). Latency to drink and total volume consumed were measured. In the marble-burying test, mice were placed in a clear polycarbonate box (18×28×13 cm) containing 5 cm of bedding with 15 marbles arranged in a 5 × 3 grid. The total number of glass marbles buried (covered by 2/3 or more) during a 30-min testing period was assessed. For the forced-swim test, animals first underwent a 15-min preswim in a glass cylinder (18 cm diameter) in ~10 cm of 25°C water. On a subsequent testing day, behavior was videotaped for 10 min and scored for immobility, climbing, and swimming. Finally, in the tail-suspension test, individual mice were suspended by the tail using adhesive tape from a plastic bar (30-cm elevation) enclosed on all but one side (8). Behavior was videotaped for 5 min, and videos were scored for total immobility time.

**Acute measurements of core body temperature**

At 5 d before drug injection, mice were individually housed and initially habituated by measurement of core body temperature using a rodent rectal probe (Physitemp Instruments, Clifton, NJ, USA). On the day of testing, core body temperature was measured 30 min after administration of indicated doses of 8-OH-DPAT (3, 9).

**Cortical neuron isolation and culture**

Cultures of dissociated cortical neurons from 1-d-old mouse pups were isolated as described previously (10). Briefly, the prefrontal cortex was dissected from P1 mouse pups, washed with HBSS, and then digested in 0.05% trypsin-EDTA (Life Technologies, Carlsbad, CA, USA) for 10 min at 37°C. Tissue was washed and resuspended in complete neurobasal medium (Life Technologies) supplemented with 2 mM glu-
tamine and B27, and cells were dissociated by repeated pipetting. Neurons were plated onto tissue culture dishes coated with poly-l-lysine (Sigma) and laminin, and the medium was replaced after 12 h. After 1 d in culture, neurons were treated with forskolin (50 μM, 15 min) followed by 8-OH-DPAT (1 μM, 15 min) and were processed for immunoblotting.

**cAMP measurements**

Isolated WT and RGS6−/− cortical neurons were first cultured in DMEM containing 1% bovine serum albumin (BSA) and 500 μM 3-isobutyl-1-methylxanthine (IMBX). After 20 min, cells were treated with forskolin (50 μM, 5 min), concurrent with 8-OH-DPAT (1 μM, 6 min) where indicated. Reactions were terminated by liquid nitrogen addition directly to the culture dishes. cAMP measurements were performed with the Direct cAMP ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA), according to the manufacturer’s protocol.

**Immunoblotting**

Whole hippocampi, dorsal striatum, and prefrontal cortex were rapidly dissected from WT and RGS6−/− mice following drug treatment, where indicated, and flash-frozen in liquid nitrogen. Tissue homogenates and cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (Sigma), quantified, and probed as previously described (11). Protein (20 μg/sample) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using standard techniques. Immunoblots were visualized using the Odyssey Imaging System with appropriate fluorescently labeled secondary antibodies (LI-COR Biosciences; Lincoln, NE, USA). Densitometric quantification of Western blots was performed utilizing ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA), and expression of indicated proteins was normalized to actin or α-tubulin-loading controls or total protein levels (phosphorylated proteins) and expressed relative to control conditions.

**Immunohistochemistry**

Formaldehyde (4%-)-perfused frozen brain sections from WT and RGS6−/− mice were processed to examine protein expression and localization. Briefly, cryosections were washed in phosphate-buffered saline (PBS), blocked with 5% BSA, and incubated overnight at 4°C with and without (control) indicated antibodies. Following washing 4 times in PBS (10 min each), sections were incubated for 1 h at room temperature with Alexa Fluor secondary antibodies (Life Technologies). Sections were visualized using confocal microscopy, as described previously (5).

**Statistical analysis**

Data were analyzed by Student’s t test or 2-way analysis of variance (ANOVA) with the Bonferroni post hoc adjustment as appropriate. Statistical analyses were performed using Prism software (GraphPad Software; La Jolla, CA, USA). Results were considered significantly different at values of P < 0.05. Values are expressed as means ± SEM.

**RESULTS**

Serotonergic deficits in brain regions comprising the cortico-limbic-striatal neuronal circuit have been implicated in anxiety and depression. We detected expression of multiple RGS6 isoforms in hippocampus and cerebral cortex of WT mice that were absent in RGS6−/− mice. The R7 family member RGS7 also exhibits robust expression in these tissues and likely contributes to the stabilization of Gβγ in the absence of RGS6 (Fig. 1A). Immunohistochemical staining of brain sections confirmed our Western blot analysis results, showing specific expression of RGS6 in prefrontal cortex and the dentate gyrus (DG), cornu ammonis 3 (CA3), and CA1 regions of the hippocampus (Fig. 1B). To confirm neuron-specific RGS6 localization, we cotained sections with the somatodendritic neuronal marker MAP2. RGS6 and MAP2 exhibit identical expression patterns in hippocampus and cortex, confirming RGS6 localization in hippocampal (CA3/CA1) and cortical pyramidal neurons (Fig. 1C). In the DG, RGS6 expression appears most prominent in the granule cell layer (Fig. 1C), a pattern we also observed in cerebellum (11).

**Figure 1.** RGS6 is expressed in the somatodendritic compartment of hippocampal and cortical neurons. A) Immunoblotting confirmed expression of multiple RGS6 isoforms, RGS6-binding partner Gβγ, and R7 family member RGS7 in hippocampus and cortex. B) Immunohistochemical staining revealed robust expression of RGS6 in the cerebral cortex and the dentate gyrus (DG), cornu ammonis 3 (CA3), and CA1 regions of the hippocampus that was lost in RGS6−/− tissue. Scale bar = 20 μm. C) RGS6 colocalizes with somatodendritic neuronal marker MAP2 in cortical neurons and hippocampal neurons of the CA1, CA3, and DG. In merged images, nuclei are stained blue using DAPI. Scale bar = 20 μm.
On the basis of these results, we hypothesized that loss of RGS6-dependent Goi2-GAP activity might contribute to the anxiolytic and antidepressant phenotype observed in mice harboring the Goi2(G148S) mutation. Therefore, we observed the performance of our RGS6−/− mice in behavior paradigms used to quantify rodent anxiety and depression-related behaviors. The open-field test is used to assess rodent response to stress from social isolation and novel environment, while simultaneously measuring locomotor activity. RGS6−/− mice exhibit mild hyperactivity (Fig. 2A), but no difference in anxiety-related behaviors, including time spent in the center of the field (Fig. 2B) and rearing (Fig. 2C). A similar lack of anxiolytic behavior in this test was observed in RGS-insensitive Goi2-knock-in mice, and, while they exhibited no increase in acute locomotor activity (3), these mice do display increased activity in the home cage (12). Conversely, RGS6−/− mice exhibit no hyperactivity in the home cage (unpublished data), indicating that the increased activity in the open field is likely a response to the novel environment.

In the elevated plus maze, which exploits the approach-avoidance conflict in rodents, RGS6−/− mice exhibit a remarkable reduction in anxiety, spending more time in the open arms (Fig. 2D) and displaying a greater number of open-arm entries (Fig. 2E). Similarly, while no difference was observed in the home cage, mice lacking RGS6 show a reduced hypo- neophagic response in a novel cage (Fig. 2F). RGS6−/− animals also consumed a larger volume of sweetened solution in both cages (Fig. 2G), an effect seen in mice treated chronically with SSRIs (7). Consistent with a reduction in anxiety, RGS6−/− mice buried fewer marbles in the marble-burying test, an approximation of obsessive compulsive disorder (OCD)-related anxiety (Fig. 2H). Depression-like behavior is assessed in rodents using models of behavioral despair, including the forced-swim and tail-suspension tests. Indeed, RGS6−/− mice show reduced immobility and climbing behavior with a concomitant increase in swimming in the forced-swim test (Fig. 2I). Interestingly, RGS6−/− mice completely phenocopy Goi2(G148S) mice, leading us to hypothesize that this phenotype results from loss of RGS6-mediated inhibition of G-protein signaling.

Treatment with WAY-100655, a 5-HT1A antagonist, rapidly reversed the antidepressant phenotype of RGS6−/− mice in the tail-suspension test (Fig. 3A), as well as the anxiolytic phenotype of RGS6−/− mice in the elevated plus maze (Fig. 3B, C). Thus, loss of RGS6 promotes anxiolytic and antidepressant behaviors by enhancing 5-HT1A-dependent signaling. In contrast, blockade of GABA2 receptors (GABA2Rs), Goi2-coupled GPCRs implicated in RGS6-dependent motor coordination (11), with antagonist SCH-50911 failed to affect performance of mice in the elevated plus maze, regardless of genotype (Fig. 3B, C). SCH-50911 reverses the ataxic phenotype of RGS6−/− mice (11). This result demonstrates that the mild ataxia observed in RGS6−/− mice (11) likely does not contribute to their performance. Furthermore, in the novelty-induced hypo- neophagia test, WAY-100655 treatment also restored the aberrant latency to drink (Fig. 3D) and solution consumption in the novel cage (Fig. 3E) observed in RGS6−/− mice to levels comparable to those observed.

Figure 2. RGS6−/− mice exhibit spontaneous anxiolytic and antidepressant behavior. A–C) RGS6 deficiency promotes mild hyperactivity (significant effect of genotype, F1,10=83.52, P<0.0001) in the open field (A) but no difference in anxiety-related behaviors, including relative time spent in the center of the field (B) or rearing (C; WT, n=13; RGS6−/−; n=19). D) RGS6−/− mice spend more time in the open arms (F; WT: F1,10=12.51, P=0.001) and solution consumption (G; F1,10=260.74, P<0.0001). H) Obsessive-compulsive disorder (OCD) behavior measured by the marble-burying test is reduced in RGS6−/− mice (WT: n=10; RGS6−/−; n=13). I) Mice lacking RGS6 show reduced depression-like behavior in the forced-swim test (WT: n=9; RGS6−/−; n=11). Data are presented as means ± SEM. *P < 0.05, **P < 0.001.
in WT mice. Thus, the reduced latency to drink and increased solution consumption seen as a consequence of RGS6 deficiency in this test also results from potentiation of 5-HT₁₄R signaling. Each of the paradigms described can be used in rodents to screen antidepressant and anxiolytic medications for predictive efficacy in human patients. Given our findings, we examined whether loss of RGS6 increases the sensitivity of mice to drugs targeting 5-HT₁₄Rs. For these experiments WT, RGS6⁻/⁻, and RGS6⁺/⁻ mice were challenged with a dose of the SSRI fluvoxamine or direct 5-HT₁₄R agonist 8-OH-DPAT insufficient to provoke antidepressant effects in WT mice. Treatment with either fluvoxamine or 8-OH-DPAT failed to provoke antidepressant effects in WT mice and had no additional antidepressant effects in RGS6⁻/⁻ mice (Fig. 3F), the latter possibly due to a maximal antidepressant phenotype in these mice. In contrast, RGS6⁺/⁻ mice, while showing no phenotypic difference from WT mice, were dramatically sensitized to the actions of both drugs (Fig. 3F). In fact, submaximal doses of both drugs decreased immobility of RGS6⁻/⁻ mice to levels observed in RGS6⁺/⁻ mice (Fig. 3F). Conversely, the hypothermic response to 8-OH-DPAT, solely mediated by 5-HT₁₄ autoreceptors in the raphe nucleus (13), occurred with equal potency and efficacy in all mice regardless of genotype (Fig. 3G). Taken together, these results indicate that RGS6 has powerful anxiogenic and prodepressant actions through its ability to terminate postsynaptic 5-HT₁₄R signaling.

We next sought to determine the molecular basis for the anxiolytic phenotype of RGS6⁻/⁻ mice. Like RGS6, 5-HT₁₄ heteroreceptors are located in the soma and dendrites of hippocampal and cortical neurons (14). No difference in 5-HT₁₄R expression levels was observed in RGS6⁻/⁻ tissue, indicating that the phenotype of RGS6⁻/⁻ mice is not due to receptor up-regulation (Fig. 4A). In brain, 5-HT₁₄R stimulation activates an array of downstream signaling, including Gβγ-mediated, phosphorylation-dependent activation of GSK3β, Akt, and MAPK and Gαo-mediated inhibition of the AC-cAMP-PKA signaling axis (15). Contrary to results obtained in Go12(1G148S)-knock-in mice (3), no difference in GSK3β phosphorylation (Fig. 4A) was detected comparing WT and RGS6⁻/⁻ tissue lysates from the cortex (Fig. 4B), hippocampus (Fig. 4C), or striatum (Fig. 4D). Similarly, no genotype-dependent changes in the levels of phosphorylated MAPK or Akt

Figure 3. RGS6 promotes anxiety and depression by inhibiting 5-HT₁₄R signaling. A) 5-HT₁₄R blockade using the antagonist WAY-100635 (WAY; 0.1 mg/kg s.c.) rescued the antidepressant phenotype of RGS6⁻/⁻ mice in the tail-suspension test (WT: n=8–9; RGS6⁻/⁻: n=8–10). Two-way ANOVA revealed a significant effect of genotype (F₁,₉₁=16.81, P=0.0003). B, C) WAY treatment, but not GABABR blockade with SCH-50911 (SCH; 30 mg/kg i.p.), reversed the anxiolytic phenotype of RGS6⁻/⁻ mice in the elevated plus maze (WT: n=8–10; RGS6⁻/⁻: n=9–13) as measured by both number of open-arm entries (B) and time spent in the open arms of the maze (C). There was a significant effect of genotype (F₁,₉₄=38.80, P<0.0001) and drug (F₂,₉₄=4.19; P=0.02) on number of open-arm entries and genotype (F₁,₉₄=36.06, P<0.0001) on time spent in the open arms by 2-way ANOVA. D) WAY treatment rescued the phenotype of RGS6⁻/⁻ mice in the novelty-induced hypophagia paradigm (WT: n=7–8; RGS6⁻/⁻: n=8–9) for both latency to drink (D) and liquid consumption (E) in the novel cage. Both genotype (F₁,₉₄=30.03; P=0.0002) and drug (F₁,₉₄=20.09; P=0.0015) affected the outcome for latency to drink by 2-way ANOVA. There was also a significant effect of genotype (F₁,₉₄=41.22; P<0.0001) and drug (F₁,₉₄=9.15; P=0.028) on volume liquid consumed in the novel cage. F) RGS6⁺/⁻ mice are sensitized to the antidepressant actions of the SSRI fluvoxamine (0.5 mg/kg) and 5-HT₁₄R agonist 8-OH-DPAT (1 mg/kg) (WT: n=8–10; RGS6⁻/⁻: n=8–10; RGS6⁺/⁻: n=9–10). Two-way ANOVA revealed a significant effect of genotype (F₂,₇₅=37.52; P<0.0001) and drug treatment (F₂,₇₅=6.51; P=0.0002). G) Neither RGS6 haploinsufficiency nor genetic ablation sensitized mice to the hypothermic effects of increasing doses of 8-OH-DPAT (WT: n=9; RGS6⁺/⁻: n=6; RGS6⁻/⁻: n=7). Two-way ANOVA revealed a significant effect of drug dose (F₂,₇₅=83.71; P<0.0001) but no effect of genotype (F₁,₇₅=0.20; P=0.65). No genotype-dependent differences in basal core temperature were detected via telemetry (unpublished results). Data are presented as means ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. WT control; †P<0.01 vs. WAY-treated RGS6⁻/⁻ mice; ‡P<0.05 vs. RGS6⁺/⁻ control; §P<0.01 vs. RGS6⁺/⁻ control.
were seen (Supplemental Fig. S1). These results show that the anxiolytic and antidepressant phenotype of RGS6−/− mice is not associated with enhanced Gβγ-mediated signaling by the 5-HT1A-R. In contrast, despite considerable heterogeneity in individual responses, we observed a 5-HT1A-R-dependent reduction in hippocampal and cortical phospho-CREB levels in RGS6−/− animals that did not occur in the striatum, where 5-HT1A-Rs are not expressed (Fig. 4). In the cortex, PKA activity, as measured by immunoreactivity of an antibody recognizing the phosphorylated PKA substrate motif (RRXT/S-P), was also reduced in RGS6−/− tissue lysates, an effect reversible by 5-HT1A-R blockade (Fig. 4B). These findings suggest that loss of RGS6 promotes 5-HT1A-R/Gβγ-mediated inhibition of the AC-cAMP-PKA axis.

Our in vivo data were recapitulated in cortical neurons isolated from WT and RGS6−/− mice. Interestingly, serum-induced phospho-CREB levels were markedly lower in cortical neurons isolated from RGS6−/− mice compared to their WT counterparts (Fig. 5A, B), possibly reflecting perpetuation of the in vivo phenotype in culture. Forskolin, a direct AC activator, resulted in robust CREB activation in RGS6−/− cortical neurons, an effect not observed in WT neurons. This suggests that increased Gβγ activity and consequent reduced AC activity underlies the lower level of CREB phosphorylation in RGS6−/− neurons. Furthermore, RGS6−/− neurons were remarkably sensitized to inhibition of CREB phosphorylation induced by 8-OH-DPAT, whereas 8-OH-DPAT had no effect on CREB phosphorylation in WT cells (Fig. 5B). To confirm a direct effect of RGS6 knockout on AC activity, we also measured forskolin-stimulated cAMP accumulation in 8-OH-DPAT-treated WT and RGS6−/− cortical neurons. Forskolin treatment resulted in cAMP generation in cells of both genotypes (Fig. 5C); however, RGS6−/−

**Figure 4.** RGS6−/− mice exhibit potentiation of 5-HT1A-R/Gβγ-dependent signaling in hippocampus and cortex. A) RGS6, 5-HT1A-R, actin, phospho-total GSK3β, and phospho-CREB, total CREB, and phospho-PKA substrate immunoblots from WT and RGS6−/− mice treated with and without the 5-HT1A antagonist WAY-100635 (WAY; 0.1 mg/kg i.p.). Data are presented in triplicate for all experimental conditions. B−D) Densitometric quantification of phospho-GSK3β, phospho-CREB, and PKA substrate phosphorylation immunoblots was performed in cortex (B), hippocampus (C), and striatum (D) (n=3–6). To confirm a significant effect of genotype on phospho-GSK3β levels in hippocampus or cortex. Two-way ANOVA revealed a significant effect of genotype on phospho-CREB levels in cortex (F1,28=7.15; P=0.009) and hippocampus (F1,28=5.12; P=0.035). There was also a significant effect of genotype (F1,28=10.46; P=0.0020) on phospho-PKA substrate immunoreactivity in cortex but not hippocampus. Neither genotype nor drug treatment affected phosphorylated protein levels in striatum. Data are presented as means ± sem. *P < 0.05, **P < 0.01.

**Figure 5.** RGS6 regulates 5-HT1A signaling through Gβγ in isolated cortical neurons. A) RGS6, α-tubulin, phospho-CREB, and total CREB immunoblots from WT and RGS6−/− cortical neurons (n=3/treatment condition) were treated where indicated with 50 μM forskolin (15 min) followed by 1 μM 8-OH-DPAT (15 min). Data are representative of triplicate experiments. B) Quantification of data from panel A by 2-way ANOVA revealed a significant effect of genotype (F1,12=20.74; P=0.0007) and drug treatment (F1,12=14.39; P=0.0006). C) cAMP levels from WT and RGS6−/− neurons (n=3/treatment condition) treated concurrently with forskolin (50 μM, 5 min) and 8-OH-DPAT (1 μM, 6 min) where indicated. Two-way ANOVA revealed a significant effect of genotype (F1,12=6.31; P=0.0145) and drug treatment (F2,12=5.12; P<0.0001). Data are presented as means ± sem. *P < 0.05, **P < 0.01 vs. WT control; #P < 0.05 vs. forskolin + 8-OH-DPAT treated RGS6−/− neurons.

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cells exhibited a more robust inhibition of cAMP accumulation in response to 8-OH-DPAT (Fig. 5C). These results indicate that RGS6 is a critical negative regulator of signaling through Goα-coupled 5-HT1A receptors in brain and isolated neurons and suggest that potentiation of 5-HT1AR-mediated AC inhibition might underlie the antidepressant phenotype of mice lacking RGS6.

In fact, direct AC activation using forskolin was sufficient to completely reverse the reduced immobility of RGS6−/− mice in the tail-suspension test (Fig. 6A), indicating that Goα-mediated inhibition of AC is exclusively responsible for reducing depression in RGS6−/− animals. As expected, on the basis of its ability to stimulate cAMP production and subsequent PKA activity, forskolin treatment resulted in elevated levels of phospho-CREB in the hippocampus and cortex (Fig. 6B, C) and PKA substrate phosphorylation in cortex and striatum (Fig. 6C, D). Notably, the reductions in both CREB and PKA substrate phosphorylation seen in RGS6−/− tissue lysates were normalized to WT levels (Fig. 6B–D), highlighting the potential importance of PKA effectors in the anxiolytic and antidepressant phenotype resulting from RGS6 loss. Thus, we propose a model whereby RGS6 counteracts the anxiolytic and antidepressant actions of 5-HT through blockade of postsynaptic 5-HT1AR-dependent AC inhibition, leading to increases in cAMP and activation of downstream AC effectors, including PKA and CREB (Fig. 7).

DISCUSSION

Here, we identify RGS6 as a critical regulator of 5-HT1AR- and AC-dependent anxiolytic and antidepressant actions. RGS6 expression is enriched in hippocampal and cortical neuron populations, 5-HT1A-expressing cells implicated in multiple mood disorders (14, 15), and loss of RGS6 potentiates 5-HT1AR-Goα-dependent AC inhibition in vivo and in isolated cortical neurons. RGS6−/− mice exhibit a spontaneous anxiolytic and antidepressant phenotype in multiple behavioral paradigms, including tests affected only by chronic antidepressant treatment. While the phenotype of RGS6 heterozygotes is indistinguishable from WT mice, partial RGS6 deficiency elicits a remarkable sensitization to the antidepressant actions of the SSRI fluvoxamine and 5-HT1AR agonist 8-OH-DPAT. These data suggest that partial loss or inhibition of RGS6 is sufficient to potentiate the actions of serotonergic drugs. Indeed, our results indicate that under physiological conditions, a single copy of the RGS6 gene is sufficient to completely prevent 5-HT-mediated signaling through postsynaptic 5-HT1ARs. Furthermore, RGS6−/− mice exhibit a level of anxiety comparable to that of WT mice treated chronically with therapeutic

Figure 6. RGS6 deficiency reduces depression by facilitating AC inhibition. A) AC activation by forskolin (7.5 mg/kg, 45 min prior to behavioral testing) rescued the antidepressant phenotype of RGS6−/− mice in the tail suspension test (WT: n=7–8; RGS6−/−: n=10–15). Two-way ANOVA revealed a significant effect of genotype (F(1,36)= 26.16; P<0.0001) and drug (F(1,36)= 4.17; P=0.0486). B) RGS6, α-tubulin, phospho-CREB, total CREB, and phospho-PKA substrate immunoblots from WT and RGS6−/− mice treated with and without forskolin (7.5 mg/kg, 45 min). Data are presented in triplicate for all experimental conditions. C–E) Den-

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5-HT1A R
drugs. While it is possible that 5-HT1ARs contribute to rodent anxiety and depression-related behaviors, two of the observations render this supposition unlikely. First, the antidepressant and anxiolytic capacity of 5-HT1AR signaling in response to endogenous 5-HT. Thus, 5-HT1AR functions as a critical countermeasure against the antianxiety and antidepressant actions of 5-HT and serotoninergic drugs. While it is possible that RGS6 deletion leads to alterations in development of the circuitry underlying anxiety and depression-related behaviors, the reasons for the observations remain unclear. We demonstrate a clear depression in PKA substrate phosphorylation in the cortex, and levels of phosphorylated CREB in both the cortex and hippocampus are greatly sensitized to subefficacious doses of 5-HT1AR-targeted drugs.

Previous work in mice expressing RGS-insensitive Goi2 demonstrated that endogenous RGS proteins block 5-HT1AR-mediated GSK3β phosphorylation (3). Given studies identifying GSK3β as an important molecular target of 5-HT (24), it was postulated that increased GSK3β phosphorylation contributed to the antidepressant phenotype of Goi2(G148S) mutants. RGS6−/− mice show an identical phenotype in the absence of increased phospho-GSK3β, suggesting that GSK3β inactivation does not cause the antidepressant phenotype resulting from loss of RGS protein regulation. Instead, RGS6 appears to selectively regulate a specific 5-HT1AR-effector pathway, namely the Goi-AC-PKA-CREB axis, without affecting other targets (e.g., GSK3β). Indeed, the ability of forskolin to reverse the antidepressant phenotype of RGS6−/− mice suggests that Gβy effectors (GSK3β, MAPK, Akt) are dispensable for the ability of 5-HT1ARs to reduce depression.

Although our results demonstrate that AC inhibition is clearly an important and essential consequence of 5-HT1AR heteroreceptor activation in reducing anxiety and depression, the exact molecular determinant of this response remains unclear. We demonstrate a clear decrease in PKA substrate phosphorylation in both the cortex and hippocampus, and levels of phosphorylated CREB in both the cortex and hippocampus are greatly sensitized to subefficacious doses of 5-HT1AR-targeted drugs.

**Figure 7.** Schematic outlining the role of RGS6 in regulation of 5-HT1AR signaling in anxiety and depression. Our results indicated that RGS6 promotes anxiety and depression by inhibiting the 5-HT1AR heteroreceptor-AC signaling axis at postsynaptic sites likely located in neurons of the hippocampus and cortex. By selectively blocking Goi-dependent AC inhibition, RGS6 facilitates cAMP accumulation and subsequent activation of PKA and CREB, which contribute to rodent anxiety and depression-related behaviors, and counteracts the actions of antidepressant and anxiolytic medications.
cortex and hippocampus of RGS6−/− mice that is normalized following 5-HT1A receptor blockade or AC activation. However, numerous signaling moieties are mobilized on AC activation, including cyclic nucleotide-gated ion channels and PKA and its substrates, which may number in the hundreds or even thousands in mammals (25). Because of its ability to promote hippocampal neurogenesis believed to contribute to SSRI efficacy (20), the role of CREB in anxiety and depression has been explored but remains controversial, as CREB appears to have prodepressant or antidepressant actions depending on the neuronal population investigated (26, 27). It is important to note that, although forskolin treatment caused a clear increase in phosphorylated CREB in the hippocampus and cortex, this ~2-fold increase was insufficient to alter the behavioral phenotype of WT animals. It seems likely, therefore, that increases in CREB activity may alleviate anxiety and depression only after prolonged stimulation, consistent with the necessity for long-term changes in neuronal architecture. In future studies, RGS6−/− mice represent a useful model system to further delineate the molecular signaling cascades responsible for the anti-anxiety and antidepressant actions resulting from 5-HT1AR-mediated inhibition of AC.

Despite the fact that other R7 RGS protein family members, including RGS7, are also expressed in hippocampus and cortex, our results clearly demonstrate that they fail to compensate for the loss of RGS6-mediated regulation of the 5-HT1A heteroreceptor-Gαi-AC signaling axis. Our observations do not, however, completely rule out a role for other members of the R7 RGS protein family, which includes RGS6, RGS7, RGS9, and RGS11, in anxiety, depression, and the actions of serotonergic drugs. Indeed, polymorphisms in the RGS7 gene are associated with panic disorder subtype and gender-biased disease susceptibility (28). However, RGS7 is unable to regulate 5-HT1AR-mediated inhibition of AC in vitro (29), indicating that RGS7 may affect this anxiety-related behavior through regulation of a different receptor signaling cascade. Mice lacking RGS9 exhibit no changes in anxiety, though their behavior has only been evaluated in the open-field test (30). Because mice lacking RGS7 or RGS11 have not been evaluated in the behavioral paradigms described in this work, it is unknown whether these R7 family members have any role in modulating anxiety and depression. Our work clearly demonstrates that RGS6 is a critical regulator of 5-HT-mediated antianxiety and antidepressant actions through its ability to inhibit Gαi-coupled 5-HT1ARs.

It is also likely that RGS6 is not the sole regulator of 5-HT1A receptor signaling in brain. Indeed, deficiency in the RGS2 gene, implicated in human anxiety disorders (31, 32), promotes anxiety and depression-related behaviors in mice by affecting expression and/or activity of 5-HT1A autoreceptors (33–35). In vivo, RGS4 overexpression inhibits 5-HT1A autoreceptors (36), and RGS4 inhibition in cortical neurons potentiates 5-HT1AR signaling (37). These studies demonstrate nondiscriminate and opposing actions of RGS4 on presynaptic and postsynaptic 5-HT1ARs. In addition, RGS4 also regulates Gαq-coupled 5-HT receptors (29), known to promote anxiety. Together, these results make predictions regarding effects of RGS4 inhibition on net behavioral outputs challenging. Recent evidence suggests that while RGS4 knockout mice exhibit no baseline difference in anxiety or depression-related behaviors, RGS4 up-regulation in the nucleus accumbens may be important in the acute and chronic actions of antidepressants (38). Thus, different RGS proteins appear to regulate 5-HT1AR populations in distinct temporal, contextual and spatial manners. RGS6 is the first RGS protein identified that appears to selectively modulate 5-HT1A heteroreceptor populations in vivo, although the exact mechanisms contributing to this heteroreceptor specificity remain unknown.

In summary, our results demonstrate that RGS6 deletion selectively enhances the actions of endogenous 5-HT at postsynaptic 5-HT1A heteroreceptors, believed to underlie the beneficial effects of serotonergic drugs, leading to spontaneous anxiolytic and antidepressant behavior. Although the exact downstream molecular determinant of antidepressant phenotype of RGS6−/− mice remains unclear, signaling cascades classically activated by the Gβγ subunit of the heterotrimeric G-protein complex appear to be dispensable, and, instead, Gαi-mediated inhibition of AC is required. In short, RGS6 functions as the gatekeeper for 5-HT-mediated anxiolytic and antidepressant actions due primarily to its ability to completely block 5-HT1AR-mediated inhibition of AC.

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Fig. S1. RGS6−/− mice show no change in phosphorylated levels of AKT or MAPK in hippocampus and cortex. (A) Phospho- and total MAPK, phospho(S473 & T308)- and total AKT, and Actin immunoblots from WT and RGS6−/− mice treated with and without the 5-HT1A R antagonist WAY-100635 (WAY) (0.1 mg/kg s.c.). Data are presented in triplicate for all experimental conditions. Densitometric quantification followed by two-way ANOVA revealed no effect of genotype or drug treatment on (A) phospho-AKT(S473), (B) phospho-AKT(T308) or (C) phospho-MAPK levels in hippocampus or cortex (n = 3). Data are presented as mean ± S.E.M.