RGS4 actions in mouse prefrontal cortex modulate behavioral and transcriptomic responses to chronic stress and ketamine.

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**Running title:** The Role of RGS4 in Stress and Ketamine Actions

**Non-standard abbreviations**

CVS: Chronic Variable Stress
mPFC: medial Prefrontal Cortex
NAc: Nucleus Accumbens
THL: Thalamus
MD-THL: Mediodorsal Thalamus
NMDA: N-methyl-D-aspartate
MDD: Major Depressive Disorder
GTPase: Guanosine Triphosphate (GTP)ase
TRAP: Translating Ribosome Affinity Purification
rs-fMRI: Resting-State fMRI
RNA-Seq: RNA Sequencing
ROI: Region Of Interest
The signal transduction protein, regulator of G protein signaling 4 (RGS4), plays a prominent role in physiological and pharmacological responses by controlling multiple intracellular pathways. Our earlier work identified the dynamic but distinct roles of RGS4 in the efficacy of monoamine-targeting vs fast-acting antidepressants. Using a modified chronic variable stress (CVS) paradigm in mice, we demonstrate that stress-induced behavioral abnormalities are associated with the downregulation of RGS4 in the medial prefrontal cortex (mPFC). Knockout of RGS4 (RGS4KO) increases susceptibility to CVS, as mutant mice develop behavioral abnormalities as early as two weeks after CVS. Resting-state fMRI (rs-fMRI) experiments indicate that stress susceptibility in RGS4KO mice is associated with changes in connectivity between the mediodorsal thalamus (MD-THL) and the mPFC. Notably, RGS4KO also paradoxically enhances the antidepressant efficacy of ketamine in the CVS paradigm. RNA-sequencing (RNA-seq) analysis of naïve and CVS samples obtained from mPFC reveals that RGS4KO triggers unique gene expression signatures and affects several intracellular pathways associated with human major depressive disorder (MDD). Our analysis suggests that ketamine treatment in the RGS4KO group triggers changes in pathways implicated in synaptic activity and responses to stress, including pathways associated with axonal guidance and myelination. Overall, we show that reducing RGS4 activity triggers unique gene expression adaptations that contribute to chronic stress disorders and that RGS4 is a negative modulator of ketamine actions.

Significance statement: Chronic stress promotes robust maladaptation in the brain, but the exact intracellular pathways contributing to stress vulnerability and mood disorders have not been thoroughly investigated. In this study, we used murine models of chronic stress and
multiple methodologies to demonstrate the critical role of the signal transduction modulator RGS4 in the medial prefrontal cortex (mPFC) in vulnerability to chronic stress and the efficacy of the fast-acting antidepressant ketamine.

INTRODUCTION

Major depressive disorder (MDD) is a severe chronic illness characterized by anhedonia, anxiety, social avoidance, pain, sleep and appetite problems, and numerous other debilitating symptoms (Manji et al., 2001; Arnow et al., 2006; Duman and Aghajanian, 2012; Labonté et al., 2017; Minkel et al., 2017). The majority of MDD patients are currently treated with monoamine-targeting antidepressants (MTAs), which have a slow onset of action, display use-limiting side effects (Lui et al., 2011; Singh et al., 2013), and show poor efficacy in a substantial subset of patients (Berlim and Turecki, 2007). In clinical settings, ketamine—which is an NMDA receptor antagonist among other actions—has rapid antidepressant effects in severe cases of MDD that are resistant to MTAs (Aan Het Rot et al., 2012). These findings have stimulated research on the mechanisms by which ketamine and other fast-acting medications affect synaptic function (Autry et al., 2011; Zanos et al., 2016; Suzuki et al., 2017). A better knowledge of the cellular and neuronal substrates involved in MDD would facilitate rational design and development of more efficacious drugs that are better tolerated and etiologically tailored.

Regulator of G protein signaling 4 (RGS4) is a potent modulator of G protein-coupled receptor (GPCR) function, with dense expression in the prefrontal cortex (PFC) and moderate expression in other mood-regulating structures such as the nucleus accumbens (NAc), dorsal raphe, and thalamus (THL) (Gold et al., 2002; Terzi et al., 2009). RGS4 binds to activated Gα subunits (primarily Gαq and Gαi) to promote their GTPase activity. It may also act as an effector antagonist for these Gα subunits (Hollinger and Hepler, 2002; Terzi et al., 2009; Sakloth et al.,
RGS4 has been shown to modulate opioid, monoamine, metabotropic glutamate, muscarinic cholinergic, and other GPCRs in the brain and periphery, and it plays important roles in physiological and pharmacological responses (Ding et al., 2006a; Schwendt et al., 2006; Han et al., 2010; Stratinaki et al., 2013b; Mitsi et al., 2015). Our earlier work revealed a potent role of RGS4 in the efficacy of a wide range of antidepressants. In fact, in mouse models of acute stress, RGS4 acts as a positive modulator of classical MTAs but as a negative modulator of ketamine and delta-opioid receptor agonists (Stratinaki et al., 2013b).

In this study, we used genetically modified mice to investigate the impact of RGS4 on vulnerability to chronic stress and identify key neuroanatomical and signal transduction mechanisms mediating RGS4 actions. Our Western blot analysis and quantitative polymerase chain reaction (qPCR) findings reveal that chronic stress in mice promotes maladaptive changes in RGS4 protein and transcript expression in the medial PFC (mPFC). Our findings from genetically modified mice suggest that blockade of RGS4 activity promotes vulnerability to stress and accelerates the development of depression-linked behaviors following chronic variable stress (CVS). These behavioral deficits are accompanied by changes in mediodorsal thalamus (MD-THL)-mPFC connectivity. Using next-generation RNA-seq and bioinformatic analyses, we demonstrate a strong influence of mPFC RGS4 on gene expression adaptations following CVS, with a subset of the observed maladaptations reversed by treatment with low ketamine doses. In summary, our studies reveal a key role of RGS4 in emotional signs of chronic stress, and highlight genes and intracellular pathways affected by RGS4 activity in the mPFC.
METHODS

Animals and drug treatments

For all behavioral and biochemical assays, we used 2-month-old male RGS4 knockout (RGS4KO) and wildtype (RGS4WT) mice, generated and genotyped as described (Han et al., 2010; Stratinaki et al., 2013b). Mice were housed in a 12-hour dark/light cycle room and were provided food and water ad libitum. All breeding, housing, and experimental procedures were performed according to the animal care and use committee of Icahn School of Medicine at Mount Sinai. For all behavioral studies, experimenters were blinded to the genotype/treatment. Ketamine (Sigma Aldrich, MO) was diluted in saline. All analyses were performed as planned and recorded in a protocol.

Acute and sub-chronic tail restraint stress

Mice were individually placed in plexiglass boxes and immobilized by taping the caudal part of the tail to the box wall for 2 h. The control group was not subjected to restraint stress or any other manipulation throughout the entire study.

Chronic variable stress

Chronic Variable Stress (CVS) was performed as published previously, but stressors were only applied once a day (Elsayed et al., 2012). Briefly, groups of male RGS4WT and RGS4KO mice were exposed to 2–4 weeks of CVS, while control animals were housed under the same conditions but were never exposed to any stressor. We used these milder stress protocols in order to determine if genetic inactivation of the RGS4 gene increases vulnerability to stress. All mice were given access to food and water ad libitum. The CVS protocol was based on a weekly schedule of the following stressors: light on for 24 h, cage tilting (45° angle), bedding removal, wet bedding, restraint for 2 h, food removal for 24 h, and water removal for 12...
h. The sequence of stressors applied was designed to maximize unpredictability. Importantly, the CVS paradigm is insufficient to produce a phenotype in WT mice.

**Western blotting assays**

Tissue from mPFC, NAc, and THL was dissected, frozen on dry ice, and subsequently lysed and sonicated in buffer containing 1% SDS and 0.1% cocktail of protease, phosphatase, and proteasome inhibitors (MG132, Sigma Aldrich, MO) as described previously (Stratinaki et al., 2013b). Membranes were incubated in solutions of the following primary antibodies: rabbit anti-RGS4 (ABT17 Millipore, MA), which has been validated in our previous publication (Stratinaki et al., 2013b) rabbit anti-GADPH (#5174, Cell Signaling, MA), rabbit anti-beta actin (#4967, Cell Signaling, MA), rabbit anti-Gβ5 C terminus (W. Simonds, National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD), and rabbit anti-RGS6/7 (Wyeth–Ayerst Pharmaceuticals, NJ). Western blotting assays were performed as previously described (Mitsi et al., 2015). After further washes in PBS-T, membranes were incubated with peroxidase-labelled goat anti-rabbit IgG (Invitrogen, MA). Bands were visualized with SuperSignal West Dura substrate (Pierce, MDN).

**Translating ribosome affinity purification (TRAP) data sets**

Reads Per Kilobase of transcript, per Million mapped reads (RPKM) values for Rgs4 were calculated from existing RNA-seq results from TRAP experiments with previously published BAC-TRAP transgenic mouse lines targeting pyramidal cells located in different laminae (L2/3, Stard8 ES2342; L5a, S100a10-bacTRAP ES691; L5b, Colgalt2-bacTRAP DU9; L6, Ntsr1-bacTRAP TS16), fast-spiking interneurons (Dlx1-bacTRAP GM520), non-fast-spiking interneurons (Htr3a-bacTRAP GM443), astrocytes (Aldh1l1-bacTRAP JD130), and endothelial cells (Abcb1a-bacTRAP ES3026), or whole cortex “input” samples (Doyle et al., 2008; Schmidt et al., 2012; Nakajima et al., 2014; Nectow et al., 2017). Three or four replicates were included.
for each TRAP data set, and 12 replicates for inputs. RNA-seq reads were mapped to annotated exons using the mouse mm10 reference genome with STAR version 2.0.0e_r291 (Dobin et al., 2013) with default settings. Raw counts were quantified using the htseq-count module of the high-throughput sequencing (HTSeq) framework version 0.6.0 using the “union” mode with default settings (Anders et al., 2015). RPKMs were calculated as $\text{RPKM} = \frac{10^9 \times \text{Count}}{\text{Library Size} \times \text{Gene Length}}$, with gene length being the maximum sum of annotated exon lengths of gene transcripts for a given gene (based on GENCODE VM9 [Ensembl 84], updated March 14, 2016). Comparative expression (fold change) was calculated as the RPKM of each immunoprecipitation (IP) divided by the mean RPKM of inputs, and values > 1 and < -1 were considered enriched or depleted, respectively.

RNA-seq and qPCR

Six groups of mice with three biological replicates per group were used for the RNA-seq study. In brief, mPFC punches were taken from individual mice (six animals per treatment group), and total RNA was isolated from pooled punches of two animals per replicate (3 replicates/treatment group), as described above, with TRIzol according to the manufacturer’s protocol. RNA was purified with RNeasy Micro columns (Qiagen, MD), and an Agilent 2100 Bioanalyzer confirmed that the RNA integrity numbers were > 8.0. The poly-A containing mRNA was purified using poly-T oligo-attached magnetic beads, and the mRNA-seq library was prepared from each pooled RNA sample using the TruSeq RNA Sample Preparation Kit v2 (#RS-122-2002) according to the instructions of Illumina. RNA-seq was performed on the Illumina HiSeq2000 machine at Mount Sinai’s Genomic Core Facility. Raw sequencing reads from mice were mapped to mm10 using TopHat2 (Kim et al., 2013). Counts of read mapping to genes were obtained using HTSeq$^3$ (Anders et al., 2015) software against Ensembl v75 mm10 annotation. Differential expression analysis was carried out using voom limma (Law et al., 2014). This analysis determined differentially expressed genes using a false discovery rate of
<5%. qPCR was performed using SYBR green (Quanta Biosciences, #95073) on an Applied Biosystems 7500 system. Reactions were run in triplicate and analyzed using the ΔΔCt method and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a normalization control.

**Bioinformatic analyses**

To identify pathway and gene functional annotations that were over-represented in our RNA-seq results, gene ontology analysis was performed using the functional annotation and clustering software tool available through the online Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009a, 2009b) Bioinformatics Resources v.6.8 (https://david.ncifcrf.gov/). We used the keywords feature (UP_KEYWORDS) for functional annotation and the term biological process (GOTERM_BP_DIRECT) for gene ontology. Only terms with p-value < 0.05 are reported. The Venn diagrams were generated using the BioVenn-web (Hulsen et al., 2008) application (https://www.biovenn.nl/index.php). To identify known and potential protein interactions between the products of the genes involved in our RNA-seq results, we used the STRING 11.0 software and database (Franceschini et al., 2013). Ingenuity Pathway Analysis (IPA) was used to identify potential activated pathways and upstream regulators (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis).

**Rank Rank hypergeometric overlap**

To evaluate global gene expression profiles, the Rank Rank Hypergeometric Overlap (RRHO) test (Plaisier et al., 2010) was performed between the six experimental groups of the study. RRHO employs a threshold-free approach to rank differentially expressed genes and determine the statistical significance of the number of overlapping genes; this method ultimately generates hypergeometric p values for the enrichment of overlapping genes for all possible rank pairs for both brain regions. The direction signed log10-transformed hypergeometric p values
were corrected for multiple comparisons according to the Benjamini and Yekutieli method (Plaisier et al., 2010).

**Forced swim test**

Forced swim testing (FST) was conducted by placing mice in 4 L beakers containing approximately 3 L tap water at a temperature of 25 ± 1 °C. An experienced independent observer blind to genotype/treatment recorded immobility times for 5.5 min, starting 30 sec after the beginning of the FST (Stratinaki et al., 2013b).

**Sucrose consumption**

Mice were singly housed with sucrose (1%) or water available for 24 h. Baseline and 24 h weights of water and sucrose solutions were taken. Sucrose consumption was determined by dividing the total sucrose consumed by the total water and sucrose consumption, multiplied by 100. Bottles were rotated at 3, 6, and 12 h to prevent side bias. Measurements were taken by a blinded observer (Krishnan et al., 2008; Descalzi et al., 2017).

**Functional magnetic resonance imaging studies**

All imaging was performed on a Bruker Biospec 70/30 7Tesla scanner with a B-GA12S gradient insert (gradient strength 440 mT/m and slew rate 3444 T/m/s). A Bruker Four-Channel mouse brain-phased array was used for all data acquisition in conjunction with a Bruker volume transmit 86mm coil. All mice were imaged under medetomidine (Dexdomitor®, dexmedetomidine hydrochloride; Orion Corporation, Espoo, Finland). First, the animal was anesthetized using isoflurane anesthesia (3% induction and 1.5% maintenance). A bolus of 0.3 mg/kg medetomidine was administered subcutaneously. Isoflurane was discontinued 5 min after the bolus administration. A subcutaneous catheter was installed, and a continuous infusion of 0.6 mg/kg/h medetomidine was used to maintain the sedation level. All mice were imaged on a
heated bed, and respiration was monitored continuously till the end of the scan. After a three-plane localizer, a field map was acquired, and the mouse brain was shimmed using the Mapshim software. A 10-min resting state blood oxygen level-dependent (BOLD) scan was acquired using the following echo planar imaging protocol: TR = 1000 ms, TE = 12 ms, FOV = 20 mm, matrix size 64 x 64, slice thickness = 0.5 mm, 20 slices, skip = 0. Six hundred frames were acquired with a total imaging time = 10 mins. After the rs-fMRI, the medetomidine was stopped, and anesthesia was switched back to isoflurane. A Diffusion tensor imaging (DTI) protocol was acquired with a Pulsed Gradient Spin Echo - EPI sequence with the following parameters: TR = 5500 ms, TE = 22.672 ms, 4 segments, 30 gradient directions with b-value = 1000 s/mm² and 5 B0’s, FOV = 25 mm, Matrix = 128 x 128, slice thickness = 0.5 mm, skip = 0, 6 averages, total acquisition time = 1 h. A T2 anatomical scan was obtained with a 3D-RARE (Rapid Acquisition with Relaxation Enhancement) sequence with a RARE factor of 8, TR = 2408 ms, TE = 56 ms, FOV = 30 mm x 27.25 mm x 30 mm, matrix size 256 x 256 x 20. At the end of the scans, medetomidine was antagonized by an injection of atipamezole (0.1 mg/kg) (Antisedan, Pfizer, Karlsruhe, Germany). Scans were transferred to an offline workstation for processing.

Connectivity measures were obtained from resting state scans. A combination of FSL (www.fmrib.ox.ac.uk/fsl) and in-house software based on Matlab (R2015b, The Mathworks Inc., Natick, MA 2000) was used to process the data. Raw time series data were slice-time and motion-corrected. Spatial and temporal filtering was then applied. BOLD scans were co-registered with the anatomical T2 scans. Region of interest (ROI) was placed in the regions of interest as identified on the T2 images. These ROIs were then applied to the BOLD time series, and the time series was then extracted.

**Statistical analysis**
Two-way ANOVA for repeated measures was utilized to examine the effects of treatment over genotype for all studies using forced swim and sucrose consumption. Post-hoc effects were revealed by the Tukey test. ANOVA or unpaired two-tailed student t-tests were utilized for comparisons between groups in qPCR and western blot analyses, as indicated in each figure. Dunnett’s multiple comparisons test was used to compare Rgs4 expression between TRAP IPs from different cell types and whole cortex inputs.
RESULTS

To identify which cell types in the mPFC express RGS4, we interrogated cell-type-specific (Doyle et al., 2008; Heiman et al., 2008, 2014) data from this region of adult male C57BL/6J mice by use of TRAP IP. We found that Rgs4 mRNA was expressed in multiple classes of pyramidal neurons but it was not present in interneurons and non-neuronal cells (Fig. 1A). While Rgs4 was expressed in all cortical layers, it was especially enriched in layer 2/3, which was also observed by in situ hybridization (ISH) from the Allen Brain Atlas in the PFC and the motor cortex (Fig. 1B).

We employed Western blot analysis to monitor RGS4 expression in the mPFC and NAc following acute and subacute stress. These experiments reveal that RGS4 expression is upregulated in both mPFC (26±10%) and NAc (18±6%) in response to acute restraint stress (AS) (Fig. 2A–C). Subacute stress (SaS) (2 h immobilization for 4 consecutive days) promotes RGS4 expression in the mPFC (29±7%) but not in the NAc (Fig. 2D–F). We next applied a murine 4-week CVS paradigm (Willner, 2016) and monitored RGS4 expression in the mPFC and NAc and THL 48 h after the application of the last stressor (Fig. 2G). In contrast to acute and subacute stress, CVS downregulates RGS4 protein in the mPFC (28±6 reduction compared to control), NAc (reduction by 6±1.3% compared to control) as well as in the THL (23±4% reduction compared to control) (Fig. 2H–J). Moreover, we observed a downregulation of Rgs4 mRNA in response to 4 weeks of CVS in the mPFC (mRNA fold change: Control: 1±0.03, 28 days CUS: 0.82±0.05. unpaired t-test, *p = 0.0148).

To determine the functional role of RGS4 in stress vulnerability, we applied a short-term CVS procedure to RGS4WT and RGS4KO mice (Fig. 3A). As shown, RGS4KO mice exposed to CVS for 2 weeks showed a trending decrease in sucrose consumption in the sucrose preference test (Fig. 3B) and a decrease in latency to immobility in the FST, while their RGS4WT counterparts show no deficits in these behavioral assays (Fig. 3C).
To better understand the role of RGS4 in functional responses to chronic stress, we applied rs-fMRI to identify changes in connectivity in naïve or 4-week CVS groups of RGS4WT and RGS4KO mice. Rs-fMRI further highlighted the role of RGS4 in chronic stress, as loss of RGS4 leads to several maladaptations in brain connectivity, including major changes in cortico-striatal and cortico-mPFC connectivity in RGS4KO CVS mice relative to stress-naïve controls. Figure 4A highlights CVS-induced increases (red) and decreases (blue) in connectivity between different mouse brain regions in RGS4KO and RGS4WT mice (Fig. 4A). We also observed genotype differences in fractional anisotropy (FA) with a diffused reduction in white matter integrity in RGS4WT naïve vs RGS4WT CVS mice, and increased white matter integrity in RGS4KO naïve vs RGS4KO CVS mice (Fig. 4B). Lastly, the rs-fMRI revealed decreased activity of functional brain networks in striatal and unilateral thalamic areas in RGS4KO CVS mice compared to RGS4KO naïve, as shown in green with substantial group differences highlighted in blue (Fig. 4C).

Our previous studies demonstrated the role of RGS4 in the mPFC in behavioral responses to ketamine (Stratinaki et al., 2013b). We show here that acute ketamine administration promoted a dose-dependent downregulation of RGS4 in the mPFC (Fig. 5A, 35±4.5% of control for 5mg/kg, 40±14.8% of control for 10mg/kg). Higher doses of ketamine (80 mg/kg i.p.) did not affect RGS4 expression, suggesting that downregulation of RGS4 upon exposure to low ketamine doses is related to the antidepressant actions of ketamine. Acute treatment with 10 mg/kg ketamine i.p. led to persistent downregulation of RGS4 in the mPFC (Fig. 5B, 91±3.5% of control at 1hr, 74±1.9% of control at 24hrs and 75±0.9% of control at 1 week) and a transient downregulation in the NAc (Fig. 5C, 67±2.4% of control at 1hr, 92±1.27% of control at 24hrs and 101±5.9% of control at 1 week) with no impact on RGS4 levels seen in the THL (Fig. 5D).
We next utilized RNA-seq to monitor the effects of CVS and ketamine treatment on gene expression in the mPFC. We applied RNA-seq analysis in order to determine gene expression patterns in the mPFC of naïve, CVS, and ketamine-treated groups of RGS4WT and RGS4KO mice. As shown in the experimental design of Figure 6A, the results were analyzed as pairwise comparisons between the different groups to identify differentially expressed genes (DEGs) (Fig. 6A). Threshold-free rank-rank hypergeometric overlap (RRHO) analysis was used to understand the impact of RGS4 gene knockout or CVS on global gene expression patterns in the mPFC. Figure 6B demonstrates that there is an overlap in the regulation of gene expression between CVS RGS4WT mice and naïve RGS4KO mice (Fig. 6B). Some of the adaptive responses triggered by CVS are common between genotypes, as suggested by the overlap in genes upregulated in response to CVS between the RGS4WT and RGS4KO groups (Fig. 6C). However, Figure 6D shows that the genes induced by CVS in the RGS4KO group are distinct from those differentially regulated in RGS4KO mice under baseline conditions (Fig. 6D). This difference is also depicted in the heatmap shown in Figure 6E, which only included genes with fold-change magnitudes $> 0.05$ and a p-value $< 0.05$. As shown in the heatmap (Fig. 6E), 2 weeks of CVS in wildtype mice produced a gene expression profile similar to that of the naïve RGS4KO, which may underlie an active stress-coping strategy. This pattern to stress is not observed in the RGS4KO groups. Indeed, 2 weeks of CVS produces gene expression adaptations in the RGS4KO group in the opposite direction from control RGS4KO mice, suggesting that CVS triggers unique maladaptations in the mutant mice that promote depression-like states. Importantly, the RNA-seq analysis verified that the 2 week CVS protocol downregulated Rgs4 (log2 fold change: - 0.389243, p = 0.04701) in RGS4WT mice, while pathway analysis using IPA predicted RGS4 as an upstream regulator after 2 weeks of CVS, associated with genes such as Acta1, Kdr, and Myh7. The Venn diagram (Fig. 6F) shows that out of 1517 DEGs in the CVS-exposed RGS4KO group, and out of 761 DEGs in the naïve RGS4KO, 138 genes are contra-regulated between the genotypes. Analysis of these genes with
the STRING protein-protein interaction predicting tool identified a highly interconnected network of proteins (Fig. 6G). Notably, none of the genes encoding these proteins is affected by CVS in the RGS4WT cohort. Further analysis using the STRING gene ontology tool revealed that the proteins of this network belong to genes implicated in myelination processes—the table in Figure 6H shows the gene ontology category (parenthesis) as well as expression direction in our RNA-seq study for these myelination-associated genes (Fig. 6H). Finally, by searching the IPA database, we identified 40 DEGs belonging to the RGS4KO CVS group previously implicated in depression and pain (Supp. Figure 1).

The heatmap in Figure 7A compares the DEGs between CVS and ketamine-treated RGS4KO and RGS4WT mice (Fig. 7A). The heatmap, as well as our behavioral observations, show that ketamine reverses the effect of stress on gene expression in the mPFC of RGS4KO mice, while it has no effect on gene expression in RGS4WT mice. The IPA upstream regulator function identified several CVS RGS4KO ketamine-treated group DEGs predicted to contribute to the gene expression changes observed in our RNA-seq data by acting as transcriptional regulators (Supp. Table 1). Moreover, IPA analysis provided additional information on the upstream pathways regulated by stress or ketamine administration (Supp. Tables 2 and 3). The Venn diagram in Figure 7B shows that more than half of the genes (467/766) affected by ketamine in the mPFC of RGS4KO mice are also affected by stress but in the opposite manner (Fig. 7B). These genes represent approximately one-third (467/1512) of the genes affected by stress in RGS4KO mice, highlighting the powerful effect of RGS4 on ketamine actions. As expected, fewer genes are contra-regulated by stress and ketamine in the RGS4WT cohort. The middle Venn diagram emphasizes the unique effect of ketamine on mPFC gene expression between genotypes. Gene ontology analysis, based on keyword terms (Fig. 7C, Grey; RGS4KO, Dark Grey; RGS4WT), reveals that the majority of pathways affected by stress and ketamine are unique in the RGS4KO group. Interestingly, predicted processes include DNA methylation, acetylation, and alternative splicing (shown in red).
We next conducted IPA comparison analysis to identify the canonical pathways affected by stress and ketamine in each genotype (Fig. 8A). Stress promotes the downregulation of several pathways in RGS4WT mice, while most of these changes are no longer observed upon ketamine treatment. Names of the pathways are shown in the full image of the pathway heatmap provided in Suppl. Figure 2. Similar to our earlier observation, our comparison analysis shows that the most robust effect is observed in the RGS4KO group treated with ketamine. In contrast, several pathways are regulated in the opposite direction upon treatment. Namely, CVS and ketamine treatment in the RGS4KO cohorts affected axonal guidance pathways, including CREB, ELPH, WNT, and Notch signaling (Fig. 8B). Figure 8C shows the genes regulated by CVS or ketamine in the RGS4KO group that are associated with WNT signaling (Fig. 8C). Separate cohorts of naïve and CVS groups of RGS4WT and RGS4KO mice were used to validate our bioinformatic analysis. As predicted in our analysis, mRNA levels for Eef2 (Fig. 8D), Gng13 (Fig. 8E), and Htr2c (Fig. 8F) are reduced by ketamine treatment in CVS groups of RGS4KO mice. Also, consistent with the results from our bioinformatic analysis, Hdac5 transcript levels are increased by 2 weeks of CVS in the mPFC of RGS4WT mice (Fig. 8G). Tspan2, a gene with a role in myelination, is upregulated in the mPFC upon knockout of RGS4, while it is downregulated by CVS (Fig. 8H). The primers used in the qPCR analysis are provided in Table 1.
DISCUSSION

Our findings reveal a fundamental role of RGS4 in maladaptive responses to chronic stress. Long-term stress causes a robust reduction in RGS4 expression in the mPFC, which contributes to the development of depression-related plasticity. Behaviorally, knockout of RGS4 increases vulnerability to prolonged stress and precipitates manifestation of functional pain signs and depression-linked behaviors within 2 weeks after CVS, a time point at which wildtype mice show minimal behavioral abnormalities. Consistent with earlier findings from our group (Stratinaki et al., 2013a), loss of RGS4 promotes the antidepressant efficacy of ketamine.

Several preclinical studies have documented the potent role of RGS4 in glutamatergic, serotonergic, noradrenergic, and dopaminergic neurotransmission. For example, RGS4 is active in a sub-group of striatal neurons and may differentially modulate dopamine D1 and D2 receptor signal transduction cascades (Taymans et al., 2003, 2004; Ding et al., 2006b). Aberrant RGS4 activity in the striatum has also been associated with addiction and Parkinson’s disease (Saugstad et al., 1998; McGinty et al., 2008; Schwendt et al., 2012; Kim et al., 2018). Furthermore, electrophysiology evidence supports a modulatory role for RGS4 in signal transduction from serotonin 5-HT$_{1A}$ receptors in the mPFC (Liu et al., 2006). This critical role of RGS4 as a modulator of monoamine receptor function was further documented by our recent studies on the impact of RGS4 on the efficacy of monoamine-targeting antidepressants in models of acute stress and neuropathic pain (Stratinaki et al., 2013b). RGS4 has been implicated in several neuropsychiatric disorders, such as schizophrenia (Mirnics et al., 2001; Paspalas et al., 2009) and Parkinson’s disease (Lerner and Kreitzer, 2012; Shen et al., 2015), but its role in MDD has not been investigated. RGS4 is present in several neural networks involved in mood, motivation, and pain modulation, including the mPFC, NAc, and THL. Based on our findings, we suggest that under short-term stress RGS4 plays a positive role as a “molecular brake” on PFC signaling pathways (Hains and Arnsten, 2008). Conversely, we
hypothesize that the reduction of RGS4 expression following chronic stress is among the maladaptive processes that promote depressive states. Earlier work by Ni and colleagues documented brain region- and modality-specific changes in Rgs4 mRNA in the hypothalamus, locus coeruleus, and cerebral cortex (Ni et al., 1999). Here, we focused primarily on the mPFC, as RGS4 is abundantly expressed in this brain region, and previous findings from our group and others have documented a potent role of mPFC RGS4 in ketamine responses in acute models of stress (Stratinaki et al., 2013b; Lur and Higley, 2018). Our TRAP findings revealed that RGS4 is expressed predominantly in layer 2/3 pyramidal neurons of the cerebral cortex. This is of great importance since it is known that repeated stress results in dendritic atrophy and spine loss in these neurons (Radley et al., 2008), effects which are proposed to be a maladaptation that promotes depressive states (Shrestha et al., 2015). Our findings further highlight the crucial interaction between RGS4-modulated pathways and ketamine actions in the mPFC. In accordance with our data, recent electrophysiology studies have demonstrated a negative modulatory effect of RGS4 in \( \alpha_2 \)-adrenergic and GABA\(_B\) receptor signaling, which is critical for the action of ketamine and modulation of glutamatergic transmission (Lur et al., 2019). Interestingly, and consistent with the literature on regional regulation of RGS proteins, ketamine uniquely affects RGS4 expression in the mPFC compared to NAc and THL. These findings emphasize that it is crucial to understand the regional role of RGS4 in stress and ketamine responses and direct our future work towards studies involving conditional knockout models. It will also be crucial to determine if similar regulation patterns are observed in female cohorts. Future studies will address whether RGS4 actions in circuits outside the mPFC also control responses to chronic stress or the actions of antidepressant medications. It will also be important to dissect the role of RGS4 in responses to chronic stress at the brain region, network, and cell-type levels. Information from such studies will help identify intracellular pathways affected by RGS4 activity as pharmacological targets for mood disorders.
Our fMRI studies further document the role of RGS4 in depression-like states by identifying major changes in connectivity in thalamocortical and thalamostriatal pathways in chronically stressed RGS4KO mice. These preclinical findings are consistent with observations from human fMRI studies, showing unilateral changes in thalamocortical connectivity in the brains of treatment-resistant MDD patients (Greicius et al., 2007; Yamamura et al., 2016). More recent preclinical work also highlighted the impact of thalamocortical circuits in responses to chronic stress (Miller et al., 2017). Importantly, an allelic variation of \( RGS4 \) has been associated with altered functional and structural connectivity in the human brain (Ding and Hegde, 2009).

Future work will dissect the role of specific projecting neurons of the THL in response to chronic stress and ketamine, as well as the function of RGS4 in each of these neuronal populations.

Clinical findings provide evidence that non-classical antidepressants, such as ketamine, are efficacious in cases of treatment-resistant depression (Murrough, 2012; Krystal et al., 2019). Acute ketamine treatment produces rapid antidepressant effects which can last for several days, presumably via actions in the mPFC, hippocampus, and other brain regions (Krystal et al., 2013, 2019; Abdallah et al., 2018). While the detailed mechanism of action is not fully understood, ketamine and its metabolites trigger fast intracellular adaptations that promote the function of molecules necessary for synaptic remodeling and recovery from pathological states (Zanos et al., 2016; Abdallah et al., 2018). Genetic ablation of the \( Rgs4 \) gene in mice enhances responses to ketamine in models of acute stress and neuropathic pain (Stratinaki et al., 2013a). In the mPFC, ketamine has been shown to promote the function of the mTOR signal transduction pathway and dendritic spine remodeling within hours after treatment (Li et al., 2010). The immediate effects of ketamine on synaptic activity and NMDA receptor currents have also been demonstrated in hippocampal tissue, where the drug promotes the activity and phosphorylation of EIF2 kinase—an action which in turn enhances BDNF expression (Monteggia et al., 2013).

Our study highlights several new intracellular pathways activated in the mPFC of RGS4KO mice after chronic stress, including the WNT, Notch, myelination, and axonal guidance networks.
Several studies, including a recent project on models of social isolation, link chronic stress to myelination changes in the mPFC (Makinodan et al., 2016; Lopez et al., 2017; Gasecka et al., 2019; Tanti et al., 2019; Issler et al., 2022). Future studies will further delineate the cellular events linking RGS4 activity to the aforementioned processes and intracellular pathways. Interestingly, earlier studies from our group have demonstrated that RGS proteins compete with AXIN2 in the brain (Gaspari et al., 2018b). It will be interesting to investigate if RGS4 in the mPFC has a similar action and to expand this work with studies on female mice.

Our RNA-seq analysis highlights genes and pathways affected by RGS4 in the mPFC. Among the DEGs in the groups exposed to chronic stress, 40 genes have documented roles in depression and pain. Strikingly, most of these genes are contra-regulated by ketamine. Several ketamine-regulated pathways, such as WNT and CREB, are known to be involved in the pathophysiology of depression (Blendy, 2006; Wilkinson et al., 2011; Dias et al., 2014; Lopez et al., 2017; Lorsch et al., 2019), and many components of these pathways are present in our DEG lists. While we speculate that RGS4 may potently affect the function of serotonin/noradrenergic receptors that affect NMDA currents, we did not test this hypothesis as the antibodies for these receptors for Western blot analysis lack selectivity. Future work will investigate how RGS4 activity in THL-mPFC projections or other mood-regulating circuits affects vulnerability to chronic stress.

Our studies provide novel insight into the regulation and function of RGS4 in a model of chronic stress and reveal a prominent role of RGS4-regulated pathways in response to ketamine, focusing on mPFC. Such intracellular pathways may provide targets for more selective and efficacious medications for MDD.
Authorship contributions


Wrote or contributed to writing the manuscript: V. Mitsi, C. Polizu, Z Farzinpour, A. Ramakrishnan, Li Shen, C.Y. Tang, E.F. Schmidt E.J. Nestler and V Zachariou

Data availability statement

All RNA-Seq datasets will be deposited at GEO, and all other data, including full images of western blots, will be available upon request.

Conflict of interest statement

The authors declare no conflicts of interest.
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Footnotes

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The authors declare no competing financial interests.
FIGURE LEGENDS

Figure 1. RGS4 expression in mPFC pyramidal neurons. (A) Relative expression (Mean ± SEM) of Rgs4 mRNA in TRAP IP mRNA compared to whole cortex input mRNA for indicated neocortical cell types. The dotted lines represent ± 2-fold enrichment or depletion of Rgs4 RPKMs (1.0 in log₂ scale; n = 12 for Whole Cortex input and n = 3-4 for TRAP IPs, Dunnett’s multiple comparisons test, **** p < 0.0001). (B) In situ hybridization (ISH) images of Rgs4 expression in mPFC (left) and motor cortex (right). Higher magnifications of boxed areas are shown at the bottom with raw ISH signal on the left and heat map expression view (EXP) on the right with red-to-blue gradient representing high-to-low expression (Lein et al., 2007). Allen Mouse Brain Atlas, mouse.brain-map.org/experiment/show/74511884. Available from mouse.brain-map.org. Allen Institute for Brain Science.

Figure 2. Regulation of RGS4 expression by acute, subacute, and chronic stress. (A) Adult male mice were subjected to 2 h of acute tail restraint stress followed by brain tissue collection. Acute stress upregulates RGS4 protein in (B) mPFC (n = 6 per group, two-tailed unpaired t-test, * p = 0.0479) and (C) NAc (n = 6 per group, two-tailed unpaired t-test, * p = 0.0292). (D) A separate cohort of male mice was subjected to subacute tail restraint stress (2 h per day for 4 days), which also resulted in an increase in RGS4 protein levels in (E) mPFC (n = 9 for Ctrl and 10 for SaS, two-tailed unpaired t-test, ** p = 0.0071), but it did not affect RGS4 expression in (F) NAc (n = 4 for Ctrl and 5 for SaS). For chronic stress studies in mice, we applied a 28-day protocol of CVS, as shown in the schematic (G). In contrast to acute and subacute stress paradigms, CVS decreased RGS4 protein expression in (H) mPFC (n = 10 per group, two-tailed unpaired t-test, ** p = 0.0013), (I) the NAc (n = 12 per group, two-tailed unpaired t-test, * p = 0.0262) and (J) the THL (n = 6 per group, two-tailed unpaired t-test, ** p = 0.0127) of adult male mice. Ctrl: control, AS: Acute stress, SaS: subacute stress. CVS: chronic variable stress.
Figure 3. RGS4KO mice develop depression-related behaviors in response to 2-week CVS. (A) To assess the impact of RGS4 in behavioral manifestations of chronic stress, we applied the CVS paradigm for 2 weeks on RGS4WT and RGS4KO mice, as shown in the timeline. (B) Genetic inactivation of RGS4 renders mice more susceptible to depression-linked behaviors as 2 weeks of CVS led to a reduction in sucrose consumption (n = 10 for Ctrl RGS4WT, n=14 for CVS RGS4 WT, n=6 for Ctrl RGS4KO and n=15 for CVS RGS4KO, two-way ANOVA, followed by Tukey’s test, * p = 0.0322) and a decrease in latency to immobility time in the FST (C) only in RGS4KO mice, (RGS4WT n=10 for Ctrl, 6 for CVS, RGS4KO n = 8 for Ctrl, 8 for CVS, two-way ANOVA followed by Tukey’s test, * p = 0.049 for Ctrl vs CVS for RGS4KO).

Figure 4. Knockout of RGS4 promotes changes in brain connectivity following CVS as assessed by resting-state fMRI. RGS4KO mice undergoing 4 weeks of CVS demonstrate several unique changes in connectivity relative to RGS4WT mice, with the most prominent effects in striatal and thalamic regions. (A) Functional Connectivity ROI analysis using rsfMRI data and correlograms indicates stronger correlations between the Orbital frontal and Cingulate Gyrus in CVS RGS4-WT (red) and reduced connectivity between multiple regions, including Hypothalamus and striatum in CVS RGS4-KO (blue). The z-score showing connectivity changes is indicated according to the colored scale. (B) Structural connectivity using DTI revealed significant differences in FA displayed in blue (<) or red (>) on top of anatomical MRI and mainly a diffused reduction in white matter integrity in RGS4WT naïve vs RGS4WT 4 weeks CVS mice (top) and increased white matter integrity in RGS4KO naïve vs RGS4KO 4 weeks CVS mice (bottom). (C) Resting-state network analysis of networks superimposed on anatomical MRI revealed decreased connectivity (voxels with substantial group differences are shown in blue) in
areas of the striatal and thalamic networks after 4 weeks of CVS in RGS4KO compared to naïve RGS4KO mice. DTI; diffusion tensor, fMRI; functional MRI, FA; fractional anisotropy.

**Figure 5. Ketamine regulates RGS4 protein levels in the mPFC.** (A) Western blot analysis demonstrates that lower ketamine doses (5 and 10 mg/kg i.p.) reduce RGS4 protein levels in the mPFC at 24 h post-treatment. A high dose (80 mg/kg) has no effect on RGS4 protein levels at 24 hrs (n = 3 per group, one-way ANOVA followed by Tukey’s test, ** p = 0.0018 for Ctrl vs 5 mg/kg and ** p = 0.0031 for control vs 10 mg/kg i.p.). (B) Ketamine treatment (10 mg/kg i.p.) leads to a reduction in mPFC RGS4 protein levels as soon as 1 h after treatment; this effect persists as it is also observed at 24 h and 1 week after the single ketamine dose (n = 3 per group, one-way ANOVA, followed by Tuckey’s test ** p = 0.0023 for Ctrl vs 24 h and ** p = 0.0034 for Ctrl vs 1-week treatment). (C) Ketamine treatment (10 mg/kg i.p.) also leads to a reduction in RGS4 expression in the NAc, but this effect is only observed at 1 h post-treatment and not at 24 h or 1-week post-treatment (n = 3 per group, one-way ANOVA followed by Tukey’s test, * p = 0.0185). (D) Ketamine treatment (10 mg/kg i.p., n = 3 per group) has no effect on RGS4 expression in the mouse THL.

**Figure 6. RNA-seq reveals distinct adaptations in gene expression in the mPFC of RGS4KO mice undergoing CVS.** mPFC tissue was collected at the end of a 2-week CVS protocol when only RGS4KO mice developed depression-linked behaviors. (A) Representative timeline for experimental paradigm. (B-D) Threshold-free comparisons of global gene expression by rank-rank hypergeometric overlap (RRHO) revealed high co-upregulation of genes between RGS4WT CVS, RGS4KO naïve, and RGS4KO CVS. RRHO revealed that RGS4KO by itself mimics many of the effects of CVS on gene expression in this brain region, while CVS causes some opposite changes in RGS4KO mice vs RGS4WT mice. (E) These findings are further illustrated with the heatmap of DEGs across the three conditions at cutoffs of
fold change > 0.5 and p < 0.05. (F) Venn diagram showing overlap of 138 genes oppositely regulated in the RGS4KO naïve vs RGS4KO CVS groups. (G) The STRING protein-protein interaction predicting tool was used to analyze these 138 genes and identify a highly interconnected hub of proteins. (H) Further GO analysis of this network revealed that the genes encoding these proteins are mainly implicated in myelination. Importantly, these myelination-associated genes are downregulated by 2-week CVS only in the RGS4KO mice, while no change is observed in the RGS4WT CVS littermates.

Figure 7. Ketamine reverses the mPFC chronic stress gene signature in RGS4KO mice. (A) Union heatmap showing DEGs regulated by CVS in RGS4KO mice relative to ketamine-treated (3 mg/kg, i.p., tissue taken 3hrs after treatment) CVS mice revealed that drug treatment reverses most of the gene changes caused by stress. On the contrary, a minor effect of ketamine on gene expression adaptations is detected in RGS4WT ketamine-treated CVS mice. (B) Separate Venn diagrams for RGS4KO and RGS4WT mice further illustrate the profoundly greater effect of ketamine on the RGS4KO group, while the middle Venn diagram reveals only 17 overlapping genes between RGS4WT and RGS4KO CVS groups treated with ketamine. (C) To gain better insight into the function of genes underlying ketamine’s actions, we conducted gene ontology analysis using keyword terms and identified very different processes between the two groups. Interestingly, a vast majority of genes in the RGS4KO group are implicated in alternative splicing and epigenetic modifications, such as methylation and acetylation (highlighted in red).

Figure 8. Ketamine triggers unique signal transduction adaptations in RGS4KO mPFC. (A) To identify signaling pathways important in antidepressant action, we used IPA and conducted a comparison analysis between each labeled condition. The dot represents an
activation z-score with an absolute value of less than 0.5. As shown, ketamine (3 mg/kg, i.p.) reverses the direction of activation in most pathways affected by stress only in the RGS4KO group. (B) Top canonical pathways modulated by ketamine in stressed KO mice include Notch and WNT/β-cat-in signaling. (C) The Wnt/β-catenin signaling pathway and its components are affected in RGS4KO mice by ketamine (right) compared to CVS (left). (D-H) Using qPCR analysis, we confirm the altered expression of the Eef2, Gng13 and Htr2c Hdac5 and Tspan2 genes in the mPFC (n = 4-7 per group, two-tailed unpaired t-test, * p = 0.0148 for Eef2, *** p = 0.0005 for Gng13, * p = 0.0348 for Htr2c and * p = 0.0316 for Hdac5. For Tspan2, * p = 0.0201, *** p = 0.0008 two-way ANOVA followed by Tukey’s test).

Table 1. information on primers used for qPCR analysis.

<table>
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<th>Primer</th>
<th>Forward 5′ -&gt; 3′</th>
<th>Reverse 5′ -&gt; 3′</th>
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<tr>
<td>MTMR1 (human)</td>
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<td>Tspan2</td>
<td>GCCGCTCGCGACCAA</td>
<td>ATACAGTGACCCGAGGC</td>
</tr>
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</table>
**FIGURE 1**

A) 

[Graph showing Log2 Fold Change (IP/input) for Rgs4 mRNA across different regions and subtypes: Whole Cortex, Pyramidal Cells (L2/3, L5a, L5b, L6), FS INs, nonFS INs, Astrocytes, and Endothelial cells.]

B) 

Immunohistochemistry (ISH) and expression (EXP) images of Prefrontal and Motor areas, showing low and high expression levels.
FIGURE 2

A) Acute Restraint Stress (AS)

B) mPFC

C) NAc

D) Subacute 4d Restraint Stress (SaS)

E) mPFC

F) NAc

G) Chronic Variable Stress (CVS)

H) mPFC

I) NAc

J) Thalamus
A) RGS4 knockout (RGS4KO) or wildtype (RGS4WT) mice

Day 1  Week 1  Week 2  Week 3

CVS  Behavioral Experiments

B) Sucrose Preference Test

% of Total Consumption

RGS4 WT  RGS4 KO

* P-value 0.0549

C) Forced Swimming Test

Latency to immobility (sec)

RGS4 WT  RGS4 KO

*
Resting-state Functional MRI:
Functional connectivity using independent component analysis

RGS4KO Naive < RGS4KO 4wk CVS ($P < 0.05$)

---

<table>
<thead>
<tr>
<th>HP</th>
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<td>Orbital Frontal</td>
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<td>Prefrontal Cortex</td>
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<td>Amygdala</td>
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<tr>
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<td>Thalamus</td>
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<tr>
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<td>Hypothalamus</td>
</tr>
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</table>
A) Ketamine (5, 10, 80 mg/kg i.p.) Dose-dependent Effect, 24hrs, **mPFC**

B) Ketamine (10 mg/kg i.p.) Time-dependent Effect, **mPFC**

C) Ketamine (10 mg/kg i.p.) Time-dependent Effect, **NAc**

D) Ketamine (10 mg/kg i.p.) Time-dependent Effect, **Thalamus**
**FIGURE 6**

**DEG (Differentially Expressed Genes) Analysis:**
- RGS4 KO Naïve [RGS4KO Naïve vs RGS4WT Naïve]
- RGS4 KO 2wk CVS [RGS4KO CVS vs RGS4KO Naïve]
- RGS4 KO Ketamine [RGS4KO CVS vs RGS4KO CVS] + Ketamine
- RGS4 WT 2wk CVS [RGS4WT CVS vs RGS4WT Naïve]
- RGS4 WT Ketamine [RGS4WT CVS vs RGS4WT CVS] + Ketamine

**Genes Implicated in Myelination**

<table>
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<tr>
<th>Gene</th>
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<th>RGS4 KO 2wk CVS</th>
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<tr>
<td>Aspa</td>
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<td>↓</td>
<td>Myelination (BP)</td>
</tr>
<tr>
<td>Cnp</td>
<td>↑</td>
<td>↓</td>
<td>Oligodendrocyte differentiation (BP)</td>
</tr>
<tr>
<td>Ernn</td>
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<td>↓</td>
<td>Myelin sheath (CC)</td>
</tr>
<tr>
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<td>↓</td>
<td>Myelination (BP)</td>
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<tr>
<td>Mog</td>
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<td>Nkx2-2</td>
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<td>Opalin</td>
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<tr>
<td>Ugt8a</td>
<td>↑</td>
<td>↓</td>
<td>Myelination (BP)</td>
</tr>
</tbody>
</table>
FIGURE 7

A) Ketamine 2wk CVS

B) RGS4 KO

2wk CVS

A) Ketamine

2wk CVS

C) RGS4 WT

2wk CVS

GO: KEYWORD

- Phosphoprotein
- Alternative splicing
- Cytoplasm
- Acetylation
- Methylation
- Transcription
- Developmental protein
- Cytoskeleton
- Cell junction
- Extracellular matrix
- SH3 domain
- Collagen
- Hydroxylation
- Laminin EGF-like domain
- Osteogenesis
- Signal
- Glycoprotein
- Disulfide bond
- Secreted
- Calcium
- Cell adhesion
- Immunity
- EGF-like domain
- Innate immunity
- Disease mutation
- Calmodulin-binding
- Pyrrolidine carboxylic acid
FIGURE 8

A) Heatmap of Canonical Pathway Regulation (IPA)

B) Top Canonical Pathways (IPA)

C) Wnt/β-catenin Signaling

D) RGS4KO

E) RGS4KO

F) RGS4KO

G) RGS4WT

H) RGS4WT RGS4KO