Protein profiling of RGS6, a pleiotropic gene implicated in numerous neuropsychiatric disorders, reveals multi-isoformic expression and a novel brain-specific isoform

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Title: Protein profiling of RGS6, a pleiotropic gene implicated in numerous neuropsychiatric disorders, reveals multi-isoformic expression and a novel brain-specific isoform

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Maity B and Stewart A, have moved to new institutions since producing the data they contributed to this manuscript. Maity B is now at the Center of Biomedical Research Lucknow, in India. Stewart A is now in the Department of Pharmacology at Florida Atlantic University in Boca Raton, FL (33431).

35 **Abstract**

36 A metanalysis identified *Regulator of G protein Signaling 6* (*RGS6*) as one of 23 loci with
37 pleiotropic effects on four or more human psychiatric disorders. This finding is significant as it
38 confirms/extends the findings of numerous other studies implicating RGS6 in CNS function and
39 pathology. RGS6 is a highly conserved member of the RGS protein family whose cellular roles
40 are likely affected by mRNA splicing and alternative domain inclusion/exclusion. Indeed, we
41 previously identified multiple RGS6 splice variants predicted to produce 36 distinct protein
42 isoforms containing either long (RGS6L) or short (RGS6S) N-terminal domains, an incomplete
43 or intact GGL domain, and 9 alternative C-termini. Unfortunately, sequence similarities between
44 the isoforms have made it difficult to confirm their individual existence and/or to determine their
45 unique functions. Here, we developed 3 RGS6-specific antibodies that recognize all RGS6
46 protein isoforms (RGS6-fl), the N-terminus of RGS6L isoforms (RGS6-L), and an 18 amino acid
47 alternate C-terminal sequence (RGS6-18). Using these antibodies, we demonstrate that
48 RGS6L(+GGL) isoforms, predominating in both mouse (both sexes) CNS and peripheral
49 tissues, are most highly expressed in the CNS. We further identify 3 novel RGS6 protein bands
50 that are larger (61, 65, and 69kDa) than the ubiquitously expressed 53-57kDa RGS6L(+GGL)
51 proteins. Importantly, we show that the 69kDa protein is a brain-specific dephospho- form of the
52 65 kDa band, the first identified phosphorylated RGS6 isoform. Together, these data begin to
53 define the functional significance behind the complexity of *RGS6* gene processing and further
54 clarifies RGS6's physiological roles by resolving tissue-specific RGS6 protein expression.

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56

57 **Significance Statement**

58 Psychiatric disorders are highly associated with polygenic variation. Consistent with this, a SNP
59 (rs2332700) in *Regulator of G protein Signaling 6 (RGS6)* is linked to autism spectrum disorder,
60 bipolar disorder, major depression, and schizophrenia. *RGS6* is a highly conserved gene
61 whose complex alternative mRNA splicing produces numerous protein isoforms with high
62 sequence similarity, hindering their functional characterization. Therefore, while aberrant *RGS6*
63 signaling and/or expression have been linked to various neuropsychiatric disorders, it is unclear
64 which isoforms are important. This study functionally delineates between the various *RGS6*
65 isoforms within mouse. We demonstrate that *RGS6* is most highly expressed in CNS,
66 characterize the predominant isoforms, and identify a brain-specific *RGS6* protein highly
67 expressed in brain regions associated with various psychiatric disorders.

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71 **Introduction**

72 Psychiatric disorders are a leading cause of worldwide disability and affect more than 25% of
 73 the population (Collaborators, 2017, Kessler and Wang, 2008). Research in this area has noted
 74 that risk is highly associated with polygenic variation (Smoller et al., 2019, Sullivan et al., 2018).
 75 Furthermore, it is now clear that psychiatric disorders are not distinct and there is significant
 76 genetic overlap between them (Anttila et al., 2018, Consortium, 2013). Highlighting this point, a
 77 recent meta-analysis conducted by the Cross-Disorder Group of the Psychiatric Genomic
 78 Consortium (2019) of 232,964 psychiatric patients and 494,162 controls revealed 23 genetic loci
 79 with significant pleiotropic effects on $\geq 4/8$ disorders studied: anorexia nervosa, ADHD, autism
 80 spectrum disorder, bipolar disorder, major depression, OCD, schizophrenia, and Tourette
 81 syndrome.

82 One of the 23 pleiotropic genetic loci described was a SNP (rs2332700) in *Regulator of*
 83 *G protein Signaling 6* (*RGS6*), previously identified in a GWAS of schizophrenic patients
 84 (Consortium, 2014), that was linked not only to schizophrenia, but also to autism spectrum
 85 disorder, bipolar disorder, and major depression. This finding is significant because of the
 86 study's magnitude and as it confirms/extends the findings of other human and animal studies
 87 implicating RGS6 in CNS function and pathology (**Fig. 1**). In particular, alterations in RGS6
 88 signaling and/or expression have been associated with: alcohol use disorders (rs11621871)
 89 (Stewart et al., 2015, Chen et al., 2017), anxiety/depression (Stewart et al., 2014), Parkinson's
 90 disease (Bifsha et al., 2014, Luo et al., 2019, Petyuk et al., 2021), Alzheimer's disease
 91 (rs4899412) (Moon et al., 2015), motor coordination (Maity et al., 2012), adult hippocampal
 92 neurogenesis (Gao et al., 2020), as well as human cataracts, mental retardation, and
 93 microcephaly (c. 1369-1 G>C) (Chograni et al., 2014).

94 RGS proteins modulate the magnitude and duration of G protein-coupled receptor
 95 (GPCR) signaling by facilitating heterotrimeric G protein inactivation, a function bestowed by
 96 their RGS domain. RGS6 is a highly conserved (**Fig. 2**) member of the R7 subfamily, which

modulates $G\alpha_{i/o}$ signaling and is distinguished by two additional domains, DEP and GGL, responsible for both membrane targeting and protein stability (Ross and Wilkie, 2000, Hooks et al., 2003). RGS6's cellular roles may also be affected by mRNA splicing and alternative domain inclusion/exclusion. Indeed, multiple RGS6 splice variants have been identified which are predicted to produce 36 distinct RGS6 protein isoforms containing either long (RGS6L) or short (RGS6S) N-terminal domains, an incomplete or intact GGL domain (-/+GGL), and 9 alternative C-termini (**Fig. 3**) (Chatterjee et al., 2003). Unfortunately, due to sequence similarity it has been difficult to confirm the existence of many of these isoforms or to determine their function. Therefore, while aberrant RGS6 signaling and/or expression have been linked to various neuropsychiatric disorders, it is unclear which RGS6 isoforms are important.

Adding further complexity are studies demonstrating RGS6 has critical physiological and pathophysiological functions outside the CNS as well. In the periphery RGS6 has been shown to: act as a critical tumor suppressor (Maity et al., 2011, Maity et al., 2013, Huang et al., 2011, Berman et al., 2004, Yang et al., 2016, Jiang et al., 2014, Luo et al., 2015, Dai et al., 2011), negatively modulate parasympathetic heart regulation (Yang et al., 2010, Wydeven et al., 2014, Posokhova et al., 2010), promote cardiovascular development (Chakravarti et al., 2017), associate with adiposity (Sibbel et al., 2011), and mediate heart-ischemic injury (Rorabaugh et al., 2017), doxorubicin-induced cardiac toxicity (Huang et al., 2011, Yang et al., 2013), as well as alcohol-induced cardiac, hepatic, and gastrointestinal damage (Stewart et al., 2015) (**Fig. 1**). Interestingly, not all of these peripheral functions (*i.e.* tumor suppression) rely on RGS6's ability to negatively regulate $G\alpha_{i/o}$ (Maity et al., 2011).

As the list of RGS6's critical physiological and pathophysiological roles continues to grow, particularly with regards to neuropsychiatric disorders, it is imperative to perform a comprehensive analysis of RGS6 isoform expression. We hypothesize such an analysis will help to clarify the functional significance behind the complexity of *RGS6* gene processing.

123 **Materials and Methods**

124 **Mice**

125 We have previously described the generation of and genotyping methods for RGS6^{-/-} mice.
 126 (Yang et al., 2010). For these studies, RGS6^{-/-} mice were back-crossed onto the C57BL6
 127 background for five generations. 3-month-old, male and female RGS6^{+/+} and RGS6^{-/-} mice were
 128 used for the experiments described in the manuscript. All animal procedures described were
 129 approved by the University of Iowa Institute for Animal Care and Use Committee.

131 **RGS6Lα1 conservation map**

132 NCBI's predicted protein sequence for human RGS6Lα1 (isoform 1-NP_001191245.1), the
 133 largest RGS6 isoform identified by Chatterjee et al. (2003) was BLASTed against the following
 134 taxids: Gorilla, Macaca, Papio, Soricidae, Mus, Oryctolagus, Sus, Camelus, Delphinus, Orcinus,
 135 Bos bovis, Ovis, Equus, Felis, Canis canis, Ailuropoda, Vulpes, Chiroptera, Loxodonta,
 136 Trichechus, Dasypodidae, and Didelphimorphia using NCBI's default settings. The sequence
 137 IDs with the highest amino acid conservation were placed in the MegAlign program (part of the
 138 DNASTAR Lasergene 12 Core Suit) and aligned using the Clustal W Method. This alignment
 139 was used to generate a circular map using the DNASTAR GenVision program.

141 **RGS6 structure homology modeling**

142 A structural model of RGS6Lα1 based on the RGS9/Gβ₅ complex structure (2PBI) (Cheever et
 143 al., 2008) was created using the Swiss-PdbViewer (Swiss Institute of Bioinformatics). Domains
 144 within the predicted RGS6Lα1 amino acid sequence that did not align with the RGS9/Gβ₅
 145 complex were modeled with an in-house algorithm (clean.swiss.pl) created by the Elcock lab
 146 (University of Iowa), which implements the Loopy program (Xiang et al., 2002). The model was
 147 visualized with VMD (Theoretical and Computational Biophysics Group, Beckman Institute,
 148 University of Illinois at Urbana-Champaign).

149 **Cell culture and transfection for protein half-life assays**

150 HEK293T cells were plated in 12 well plates at 3.5×10^5 cells/well and were cultured in High
151 Glucose Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 10%
152 fetal bovine serum (Gibco® 26140), 100 units/mL penicillin (Gibco® 15140), and 100µg/mL
153 streptomycin (Gibco®15140). 24hrs later cells were transiently co-transfected using the
154 Lipofectamine 2000 system (ThermoFisher Scientific® 116680) with a pcDNA3.1-HA vector
155 containing Gβ₅ (Gβ₅-HA) (gifted by Songhai Chen), a pEGFP vector containing R7BP (R7BP-
156 GFP) (gifted by Martemyanov, Scripps, FL), and a pcDNA3.1 vector containing RGS6 (1:1:2
157 ratio, total DNA =1µg/well). Transfection media was exchanged for new supplemented DMEM
158 media at 6hrs following transfection. 24hrs post-transfection, supplemented DMEM was
159 removed, and cells were cultured in serum free supplemented DMEM containing 100µM
160 cycloheximide (Sigma C6255) for 0, 2, 4, 6, 12, and 24hrs.

161

162 **Antibody generation**

163 Three different polyclonal RGS6 antibodies were used in this study. Antibodies to the N-
164 terminal domain of RGS6L (RGS6-L) were generated with a synthetic peptide immunogen
165 corresponding to residues 1-19 (MAQGSGDQRAVGVDPEESC-COOH) of RGS6L
166 (Biosynthesis Inc) (Chatterjee et al., 2003). Antibodies to the unique splice forms of RGS6 that
167 retain exon 18 sequences (RGS6-18) were generated (Biosynthesis Inc.) with a peptide
168 immunogen corresponding to 14 amino acids in this region (-CKPESEQGRRTSLEK)
169 (Chatterjee et al., 2003). Antibodies to full length RGS6L (RGS6-fl) were generated (Elmira
170 Biologicals) to recombinant RGS6L following its expression, solubilization, and purification from
171 inclusion bodies in bacteria.

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175 **Western Blot**

176 *Cell Culture:* Media was aspirated off cells and 100µl of ice cold RIPA buffer (150mM NaCl, 1%
177 NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50mM Tris-HCl: pH 8)
178 containing protease (Roche 11836170001) and phosphatase (Sigma p5726) inhibitors was
179 added to each well. Cells and RIPA were then placed in pre-chilled tubes, vortexed for 30sec,
180 incubated on ice for 5min, and centrifuged at 8000rpm (4°C) for 10min. Supernatant was
181 transferred to tubes containing 4x SDS PAGE Sample Buffer and boiled for 5 minutes before
182 loading onto gels.

183 *Tissues:* Tissues were prepared for western blot analysis as previously described (Yang et al.,
184 2016).

185 Western blots were probed with home-made rabbit anti-RGS6-specific antibodies
186 (RGS6-L, RGS6-18, and RGS6-fl; 1:1,000-2,000), rabbit anti-Gβ5 (a gift from Dr. William F
187 Simonds, 1:1000 dilution)(Zhang et al., 1996), rabbit anti-RGS7 (a gift from Vladlen Z. Slepak,
188 1:1000 dilution)(Levy et al., 1999), rabbit anti-RGS9-2 (a gift from Steve Gold, 1:1000
189 dilution)(Psifogeorgou et al., 2007, Psifogeorgou et al., 2011), mouse-anti-RGS11 (a gift from
190 Jason Chen, 1:1000 dilution)(Chen et al., 2010), as well as commercial mouse monoclonal anti-
191 α-Tubulin (CalBiochem, CP06-100UG, 1:5000 dilution) and rabbit anti-actin (Sigma A2066,
192 1:1000), primary antibodies. LI-COR Odyssey goat anti-mouse (926-32220 and 926-32210)
193 and goat anti-rabbit (926-32211 and 926-68071) secondary antibodies were used at a 1:10,000
194 dilution for protein visualization with the Odyssey system.

195

196 **Immunohistochemistry**

197 Mice were perfused with a saline solution at 1 mL/min rate for 5 min, followed by infusion of a
198 freshly made fixative solution (4% paraformaldehyde, 23 mM NaH₂PO₄, 77 mM Na₂HPO₄) at 1
199 ml/min rate for 15 min. Tissues were collected and soaked in the fixative solution (4%
200 paraformaldehyde, 23 mM NaH₂PO₄, 77 mM Na₂HPO₄) overnight. After fixation, tissues were

201 embedded in paraffin and sectioned. Tissue sections of RGS6^{+/+} and RGS6^{-/-} mice were
 202 processed to examine expression of RGS6 as we described previously (Maity et al., 2011).
 203 Briefly, sections were dewaxed in xylene, treated with a graded series of alcohol solutions,
 204 immersed in 3% hydrogen peroxide to block endogenous peroxide activity, blocked with 5%
 205 bovine serum albumin and then incubated overnight at 4°C with rabbit anti-RGS6-fl antibody.
 206 Following washing (3 × 10min) in PBS, sections were incubated for 1h at room temperature with
 207 peroxidase-conjugated secondary antibodies (Cell Signaling, 7074). A positive reaction was
 208 detected by exposure to stable diamin-obenzidine (General Biosciences Corporation) for 3min.
 209 The sections were counterstained in Harris hematoxylin and observed under the microscope.
 210 This is true for all tissues samples except heart. For mice hearts, frozen sections were blocked
 211 for 1 hour at 4°C in blocking buffer, containing 10% goat serum, 0.3% triton X-100, 80.4 mM
 212 Na₂HPO₄, 19.0 mM NaH₂PO₄, pH7.4, and then incubated overnight at 4°C with rabbit anti-
 213 RGS6-fl antibody. Following washing (3 × 10min) in the blocking butter, sections were
 214 incubated for 1h at room temperature with Alexa-conjugated secondary antibodies (Fisher
 215 scientific, A11011).

216

217 **Bioinformatic prediction of RGS6 phosphorylation sites**

218 Putative phosphorylation sites in RGS6Lα1 were identified using the Group-based Prediction
 219 System (GPS) 3.0 from Cuckoo Workgroup (Hubei, China). This software uses hierarchical
 220 grouping of protein kinases to identify possible kinase- or family-specific phosphorylation sites in
 221 each protein sequence based on a metric measuring similarity of the protein sequence to known
 222 phosphorylation site motifs. The software considers each specific serine/threonine/tyrosine
 223 residue and 7 amino acids up and downstream of that residue in generating a prediction score,
 224 a measure of the likelihood of peptide phosphorylation. In our analysis the threshold was set to
 225 high and predicted site/kinases were considered if their GPS prediction score was at least 1
 226 above the cutoff value defined by the software ($\Delta\text{Score} \geq 1$). Phosphorylation sites and

corresponding kinases meeting these criteria are listed in **Fig. 7-1**. Subsequently, a diagram depicting each putative RGS6 phosphorylation site and the corresponding kinase with the highest prediction score was constructed (**Fig. 7A**).

Phosphatase/kinase assays

Analysis of RGS6 phosphorylation and de-phosphorylation was assayed in RGS6^{+/+} and RGS6^{-/-} whole brain tissue lysates. Tissues were homogenized in the absence of phosphatase inhibitor cocktail. Lysates were diluted to a final concentration of 2µg/µl in 1X NEBuffer Pack for Protein MetalloPhosphatases (New England BioLabs; Ipswich, MA, USA). 100 units λ phosphatase (New England BioLabs) were added to each reaction as indicated. For kinase assays, lysates were supplemented with 2 mM ATP and 1mM MgCl₂ required for enzyme activity, and with or without activators of PKA, PKC, or Ca²⁺-stimulated kinases as shown in **Fig. 7D**. 30mM NaF was added to reactions lacking λ phosphatase and in kinase reactions to inhibit endogenous phosphatases. Reactions were incubated at 30°C for 20min and terminated through the addition of 1X sample buffer. Samples were subjected to SDS-PAGE and immunoblotting as described above.

Experimental Design and Statistical Analysis

All graphical data are expressed as mean ± S.E.M. All sample sizes reported are based on conservative power analyses estimating β = 0.2 (80%) with statistical significance being evaluated against α = 0.05. Effect sizes were estimated using Cohen's d formulation prior to calculating power analyses. Significant differences within tissue expression data (**Figs. 5B, 8A, 9A and Supp. Figs. 2-4**) were analyzed via two-way ANOVA with Fisher's LSD *post-hoc* adjustment. Significant differences within immunoreactivity data (**Figs. 7C and E**) were also analyzed via two-way ANOVA with Fisher's LSD *post-hoc* adjustment. All data were analyzed using XLSTAT software.

253 Results

254 Development and characterization of RGS6 antibodies confirms *in vivo* expression of 255 RGS6L and identifies novel RGS6 protein bands

256 We previously cloned 36 distinct RGS6 mRNA splice-forms, from a human brain cDNA library,
257 which were predicted to produce long (RGS6L) and short (RGS6S) RGS6 protein isoforms with
258 an incomplete or intact GGL domain (-/+GGL) and 9 alternative C-termini (**Fig. 3A**) (Chatterjee
259 et al., 2003). In this study, we endeavored to confirm the existence of these RGS6 isoforms *in*
260 *vivo*. Therefore, we developed three antibodies, against: the whole RGS6L protein (RGS6-fl),
261 the N-terminus of RGS6L protein isoforms (RGS6-L), and an alternative 18 amino acid
262 sequence found in the C-terminus of 56% of RGS6 isoforms (RGS6-18). **Figure 3B** shows the
263 N-terminal sequence of RGS6 to which the RGS6-L antibody was generated and the lack of
264 sequence conservation in this region with other R7 subfamily members. **Figure 3C** shows the
265 alternatively spliced 18 amino acid sequence to which the RGS6-18 antibody was generated.
266 Because the RGS6-fl antibody was generated to recombinant RGS6L α 2 this antibody likely
267 targets multiple epitopes on RGS6 proteins. All antibodies were generated in rabbit against the
268 human protein or its sequences that are conserved in mouse. We examined the ability of these
269 three antibodies to detect native RGS6 protein isoforms by comparing western blots of whole
270 brain lysates derived from RGS6^{+/+} (WT) to RGS6^{-/-} (KO) mice (Yang et al., 2010) in order to
271 identify RGS6-specific bands. **Figure 4A** shows representative western blots from our studies.

272 Anti RGS6-fl recognizes three RGS6-specific bands identified by the magenta, blue and
273 green arrows (**Fig. 4A**, left panel). The most abundant of these immunoreactive bands
274 (magenta arrow) has a molecular weight of 53-57kDa, a size range corresponding to RGS6L
275 protein isoforms containing the GGL domain, required for protein stabilization (RGS6L(+GGL),
276 **Fig. 4-1**) (Chen et al., 2003, Cheever et al., 2008, Chatterjee et al., 2003). In addition, RGS6-fl
277 recognizes 61kDa (blue arrow) and 69kDa (green arrow) bands that are larger than previously
278 described RGS6 isoforms. We show below that the 69kDa isoform represents a novel brain-

specific form of RGS6L (**Fig. 5 and 8**). Finally, the RGS6-fl antibody also detected a 58kD band (**Fig. 4A**, asterisk) in both RGS6^{+/+} and RGS6^{-/-} brain lysates, demonstrating this is not a RGS6 protein isoform. We confirmed by immunoblotting using an RGS7 antibody that this band represents RGS7 (not shown), the R7 subfamily member most closely related to RGS6. This was unsurprising given that RGS6-fl was generated to recombinant RGS6L.

Interestingly, while protein bands corresponding to RGS6L(+GGL) were readily apparent when using the RGS6-fl antibody isoforms lacking the GGL domain (RGS6L(-GGL), 49-52kDa, **Fig. 4-1**) were not (**Fig. 4A**, left panel) even with long blot exposures (not shown). Because the RGS6-fl antibody can detect RGS6L(-GGL) isoforms expressed in HEK 293 cells (**Fig. 4B**), the apparent absence of these isoforms *in vivo* is likely due to their relative instability compared to RGS6L(+GGL) isoforms, as suggested by cycloheximide chase experiments in HEK cells (**Fig. 4C and D**), and/or low abundance when compared to RGS6L(+GGL) isoforms. Interestingly, the RGS6-fl antibody also did not detect RGS6S(+/-GGL) proteins (34-41kDa, **Fig. 4-1**) in mouse brain (**Fig. 4A**, left panel), even though RGS6S transcripts were originally cloned from a human brain cDNA library (Chatterjee et al., 2003). As with the RGS6L(-GGL) isoforms, the inability to detect the RGS6S isoforms is not due to a lack of antibody specificity because anti-RGS6-fl readily detects RGS6S(+/- GGL) proteins following expression in HEK293 cells (**Fig. 4B**). Therefore, lack of RGS6S isoforms *in vivo* may be due once again to their relative instability, suggested by cycloheximide chase experiments (**Fig. 4C and D**), and/or low their abundance when compared to RGS6L isoforms.

As the RGS6-fl antibody recognized not only RGS6, but also RGS7, a second antibody, RGS6-L, was generated against the first 19 amino acids (MAQGSGDQRAVGVDPEES) of the RGS6L isoforms, a sequence that shows minimal conservation across the R7 subfamily (**Fig. 3B**). The caveat to the RGS6-L antibody is that it does not detect RGS6S(+/-GGL) isoforms (**Fig. 4B**) which have a truncated N-terminus (**Fig. 3A**). However, given that the RGS6-fl antibody did not detect endogenous RGS6S isoforms in mouse brain (**Fig. 4A**, left panel), or in

any other mouse peripheral tissues tested (data not shown), use of the RGS6-L antibody was deemed an acceptable alternative strategy. Subsequent western blot validation of the RGS6-L antibody revealed that it detects the same three RGS6 protein bands (magenta, green, and blue arrows) as the RGS6-fl antibody, but, unlike the RGS6-fl antibody, it does not detect RGS7 (asterisk, **Fig. 4A**, middle panel). Again, the most abundant immunoreactive band detected with both RGS6L and RGS6-fl antibodies is the 53-57 kD band (magenta arrow), corresponding to RGS6L(+GGL) isoforms (**Fig. 4-1**), followed by the brain-specific 69 kD band (green arrow) and then the 61kD band (blue arrow, **Fig. 4A**, left and middle panels). No other RGS6 proteins are detected by these two antibodies.

While the RGS6-L antibody eliminates detection of RGS7, it does not allow for easy delineation between the nine RGS6L(+GGL) isoforms, as they fall within a narrow molecular weight range (53-57kDa, **Fig. 4-1**). Therefore, a third antibody, RGS6-18, was generated against the alternative PESEQGRRTSLEKFTRSV amino acid sequence (**Fig. 3C**) encoded by exon 18 present near the C-terminus of 56% of RGS6 isoforms (**Fig. 3A**, dark grey box starting at amino acid 456). As the RGS6-L and RGS6-18 antibodies were both made in rabbit, it was not possible to directly compare their banding pattern in a single blot. However, western blot analysis using RGS6-18 shows strong immunoreactivity of the 69kDa brain-specific band (green arrow) and the 53-57kDa band (magenta arrow) corresponding to RGS6L(+GGL) isoforms (**Fig. 4A**, right panel). The RGS6-18 antibody also recognizes RGS7 (asterisk, **Fig. 4A**, right panel) as a portion of the alternative amino acid sequence (RRTSLEKFTRSV) is present ($\leq 75\%$ conservation) in a subset of RGS7 isoforms.

In summary, western blot analysis of RGS6 isoform expression in mouse brain tissue reveals that two of the antibodies, RGS6-fl and RGS6-18, also detect RGS6's closest evolutionary relative, RGS7. However, the RGS6-L antibody generated against the N-terminal sequence unique to RGS6L isoforms eliminates this problem. Further analysis revealed that while RGS6 proteins with molecular weights corresponding to RGS6L(+GGL) isoforms were

readily detectable, RGS6L(-GGL) and RGS6S(+/-GGL) isoforms were not, possibly due to their relative instability or low abundance.

There are two remaining points of interest that arose from these initial cell culture and mouse tissue experiments. First, we discovered that overexpression of RGS6L(+/-GGL) and RGS6S(+/-GGL) transcripts in culture results in the expression of several protein bands, detected by the RGS6-fl antibody, that are not only equivalent in size but also smaller than the predicted protein molecular weights (**Fig. 4B**, yellow arrows and **4C**,). Subsequent analysis of both transcript sequence and protein band molecular weights suggest that the small bands are likely RGS6 protein isoforms produced through alternative start sites. These alternative start sites are present in all four overexpressed transcripts, and thus similar low molecular weight bands are produced by each. As these alternative start sites exist in exons 8 and 10 (yellow stars, **Fig. 4-2**), present in all RGS6L and RGS6S transcripts, we would predict that these low molecular weight RGS6 proteins may be expressed in any tissue expressing RGS6. Second, western blots of mouse whole brain lysate revealed at least two additional RGS6 protein bands that were larger (61 and 69kDa, blue and green arrows, respectively) than all previously described isoforms (**Fig. 4A**, left and middle panel). Both protein bands were detected by the RGS6-fl and RGS6-L antibodies, but only the 69kDa protein band was detected by the RGS6-18 antibody (**Fig. 4A**, right panel). From these data, the significance of these bands was uncertain. They are bona fide RGS6 protein isoforms as they were not detected in RGS6^{-/-} mice, but whether they represent novel RGS6 isoforms or post-translational modified proteins remained unclear.

RGS6L(+GGL) is most highly expressed in CNS, but is also expressed in several peripheral tissues

As the RGS6-L antibody does not recognize RGS7, we used it to conduct a comprehensive western blot analysis of RGS6L expression in 31 tissues throughout the mouse body (**Fig. 5**).

357 To standardize the westerns and allow for quantitative analysis of RGS6 expression across
 358 tissues, one RGS6^{+/+} whole brain lysate was included as a control/constant denominator in
 359 every blot. These blots, and the subsequent densitometric analysis, revealed that RGS6L is
 360 significantly expressed in all CNS tissues analyzed, including, but not limited to the: cerebral
 361 cortex, olfactory bulb, midbrain, cerebellum, spinal cord, and retina (**Fig. 5**). Furthermore,
 362 RGS6L isoforms were also found at significant, albeit lower levels (**Figs. 5 and 5-1**) in two
 363 peripheral mouse tissues (heart and bladder). Finally, while statistical significance was not
 364 achieved due to small sample size, tissue variability, or low level of expression, RGS6 protein
 365 bands were also identified in lung, kidney, prostate, heart, omental fat, stomach, intestines, and
 366 breast (**Fig. 5**). This RGS6 expression profile corresponds well with previous reports describing
 367 the physiological and pathophysiological roles of RGS6 (**Fig. 1**).

368 To compliment this broad expression profile, we conducted an immunohistochemical
 369 analysis of RGS6 distribution (**Fig. 6**) in tissues found to express RGS6 protein bands via
 370 western (**Fig. 5**). The RGS6-fl antibody was used for this analysis, instead of the RGS6-L
 371 antibody, as it proved to stain tissues more reliably (data not shown). Furthermore,
 372 simultaneous staining of both RGS6^{-/-} and RGS6^{+/+} tissues demonstrated that, unlike with
 373 western blot, the RGS6-fl antibody does not detect RGS7 proteins in intact tissues (**Fig. 6**). In
 374 the CNS, this analysis revealed that RGS6 is expressed ubiquitously across the cortical layers
 375 and confirmed what we had previously reported in the cerebellum (Maity et al., 2012), that
 376 RGS6 is expressed predominantly by neurons in the granule cell layer and Purkinje neurons.
 377 Likewise, there is a predominant neuronal expression of RGS6 in the spinal cord and olfactory
 378 bulb, as well as the midbrain, the latter finding confirming our previous evidence that RGS6 is
 379 expressed in midbrain dopaminergic neurons (Bifsha et al., 2014, Luo et al., 2019). Finally, in
 380 the retina, RGS6 proteins are highly expressed in ganglion cells as well as retinal pigmented
 381 epithelium and to a lesser extent in bipolar cells, rods, and cones. Moving to the periphery,
 382 robust RGS6 expression is detected throughout the walls of the heart, in omental fat cell

383 plasma, and in epithelial cells of lung, bladder, breast, intestines, stomach, and kidney. A lower
384 RGS6 expression is also seen in connective/muscular tissues adjacent to epithelial cells (**Fig 6**).

385 Finally, an in-depth analysis of the RGS6 expression profile blots also elucidated the
386 most prevalent RGS6L isoforms. Consistent with what had been seen earlier in whole brain
387 lysates, the molecular weight of the protein bands observed suggested that RGS6L(+GGL)
388 isoforms predominate (magenta arrow, 53-57kDa) in all tissues examined. Furthermore, it
389 became apparent that, in addition to the large 61 and 69kDa bands (blue and green arrows,
390 respectively), the RGS6-L antibody also detected a third novel protein band of ~65kDa (orange
391 arrow) in RGS6^{+/+} and RGS6^{+/-}, but not RGS6^{-/-}, mice. This band, while faint, was clearly visible
392 in most CNS tissues blots with longer exposure times. Examination of the expression profile for
393 all three bands revealed that while the 61kDa band is expressed in multiple tissues (brain,
394 omental fat, breast, heart, stomach, intestine, lung, and bladder) the 65 and 69kDa bands are
395 brain-specific (**Fig. 5**). Once again, the significance of these findings, whether these bands
396 represented novel isoforms or post-translationally modified proteins, remained unclear.
397 However, considering RGS6's association with various neuropsychiatric disorders, we were
398 very intrigued by the discovery that two of these novel RGS6 protein bands were brain-specific.
399

400 **The novel, 65kDa brain-specific RGS6 is a phosphorylated protein**

401 Phosphorylation is a common form of post-translational modification. Indeed, the R7 family
402 members, RGS7 and RGS9, have previously been shown to be phosphorylated (Benzing et al.,
403 2002, Benzing et al., 1999, Sokal et al., 2003, Balasubramanian et al., 2001, Garzón et al.,
404 2005, Ibi et al., 2011). There is also evidence from large-scale proteomic screens of the murine
405 brain that supports the existence of RGS6 phospho-peptides with modifications on S244, S437,
406 S459, and S490 of RGS6Lα1 (Safaei et al., 2011, Trinidad et al., 2012, Huttlin et al., 2010).
407 Furthermore, group-based prediction of protein phosphorylation using GPS 3.0 software
408 indicates that there are potentially 46 threonine, serine, and tyrosine residues in the RGS6Lα1

409 protein that at serve as kinase substrates (**Fig. 7A** and **Fig. 7-1**). Several of these residues
 410 were also identified as sites of potential phosphorylation via a second, similar program,
 411 PhosphoNET, as well (**Fig. 7-1**). Therefore, we hypothesized that the large novel RGS6L
 412 protein bands may represent phospho-proteins and subsequently performed an *in vitro*
 413 phosphatase assay using protein lysates prepared from the whole RGS6^{+/+} and RGS6^{-/-} mouse
 414 brains. This assay revealed that addition of λ phosphatase to the lysate mixture eliminated the
 415 65kDa RGS6L protein band, but not the 61 and 69kDa RGS6L species (**Fig. 7B**). This assay
 416 suggests that the 65kDa RGS6L protein band (orange arrow) represents a brain-specific
 417 phospho-protein.

418 Further experiments revealed that the 65kDa and 69kDa RGS6 isoforms represent
 419 interconvertible phospho- and dephospho-forms of RGS6, respectively. First, in support of this,
 420 analysis of the effects of phosphatase treatment on RGS6 isoforms revealed that phosphatase-
 421 induced loss of the 65kDa band was accompanied by an increase in the 69kDa band (**Fig. 7C**).
 422 This suggested that dephosphorylation of the 65kDa band did not result in its disappearance,
 423 but instead promoted an upward shift in its electrophoretic mobility to 69kDa. Though
 424 phosphorylation does not change the electrophoretic mobility of most proteins, it can lead to an
 425 upward or downward shift in mobility depending upon the protein. Our results suggested that
 426 dephosphorylation of the 65kDa form produced an upward mobility shift. Noteworthy examples
 427 of proteins showing upward mobility shifts upon phosphorylation include cyclin-dependent
 428 kinase-2 (Cdk-2) (Gu et al., 1992), Cdk1 (Larochelle et al., 1998), and Cdk-7 (Garrett et al.,
 429 2001). To confirm this, we performed kinase reactions in brain extracts with ATP and various
 430 kinase activators. **Figs. 7D** and **7E** show that incubation of extracts with ATP and Ca^{2+}
 431 promoted an increase in the 65kDa RGS6 band and a decrease in the 69kDa RGS6 band.
 432 These findings corroborate our evidence from **Figs. 7B** and **7C** that the 65kDa band is a
 433 phosphorylated form of RGS6 and that dephosphorylation leads to an upward mobility shift to
 434 the 69kDa band.

435 The predicted size of the largest RGS6 isoform shown in **Fig. 7A** is 56.5kDa (**Fig. 4-1**).
436 Our studies shown in **Figs. 7B-7E** show that the 53-57kDa and the 61kDa isoforms are not
437 affected by phosphatase treatment or by incubation with ATP and kinase activators. Yet, all
438 known members of the RGS6 protein family have some or all of the sequence elements shown
439 in **Fig. 7A**. It is possible that the expected additional protein sequence in the novel brain-
440 specific 69kDa possesses the site for Ca^{2+} -dependent phosphorylation. Alternatively, it may
441 possess sequence elements that confer substrate availability of sequences present in all/most
442 RGS proteins shown in **Fig. 7A**. It is also possible that the 53-57kDa isoforms do not undergo
443 mobility shifts upon phosphorylation/dephosphorylation and that the 61kDa isoform represents a
444 novel RGS6 isoform whose transcript was not identified in the initial cloning effort (Chatterjee et
445 al., 2003).

446
447 **The novel, 69kDa brain-specific RGS6 isoform is highly conserved and significantly**
448 **expressed in all regions of the brain analyzed**

449 We found that the 69kDa dephospho- form of RGS6 was generally easier to detect and quantify
450 in various brain regions than the 65kDa form, possibly due to dephosphorylation of the 65kDa
451 isoform following sacrifice and brain dissection. The function of these isoforms in brain is
452 unclear, but a cursory analysis hints at their importance. First, quantification of the 69kDa band
453 demonstrates that it is significantly expressed, in all RGS6^{+/+} mouse brain tissues assayed
454 (**Figs. 8A and 8-1**), although its expression level appears to be consistently lower than that of
455 the 53-57kDa RGS6L protein isoforms (**Figs. 7B and 7D**). Given its exclusive expression in
456 brain tissue we hereafter refer to the 69kDa isoform as RGS6B (and the 65kDa form as p-
457 RGS6B). Second, RGS6B appears to serve an important evolutionary function as western blot
458 analysis reveals that it is not only expressed in mouse brain tissue, but also human brain tissue
459 (**Fig. 8B**, green arrow).

460

461 **RGS6 is the only R7 family member expressed in lung, omental fat, bladder, stomach,**
 462 **and intestine**

463 As mentioned earlier, the western blot profile of RGS6L protein expression (**Fig. 5**) was
 464 consistent with the literature regarding RGS6's physiological and pathophysiological roles (**Fig.**
 465 **1**). However, previous analysis of RGS mRNA expression profiles indicate that there is often
 466 overlap in the expression of several RGS's within a single tissue. For example, *in situ*
 467 hybridization (Gold et al., 1997) and RT-qPCR analyses (Larminie et al., 2004) demonstrated
 468 that the entire R7 RGS subfamily is expressed in the brain. Therefore, to determine whether, in
 469 tissues where it is expressed, RGS6 is the primary R7 subfamily member present we re-probed
 470 the RGS6-L western blots (**Fig. 5**) with an antibody against $G\beta_5$ (**Fig. 9**). $G\beta_5$ expression was
 471 found to be significantly higher in brain and retina vs other tissues (**Fig. 9-1**) as observed for
 472 RGS6L (**Fig. 5-1**).

473 $G\beta_5$ is an atypical $G\beta$ subunit that forms an obligate dimer with all R7 family members
 474 through interaction with their GGL domain (**Fig. 3**), which is structurally homologous to
 475 conventional $G\gamma$ subunits. Interaction between $G\beta_5$ and R7 family members is obligatory, loss
 476 of either interacting partner causes destabilization of the other (Chen et al., 2003). Emphasizing
 477 this point, in 2008 Cheever and colleagues solved the crystal structure of RGS9 and showed it
 478 in complex with $G\beta_5$. Furthermore, this crystal structure revealed that the interaction between
 479 RGS9's GGL domain and $G\beta_5$ mirrors the orientation and interaction of the conventional $G\beta\gamma$
 480 dimer. This interaction orientation is believed to be maintained throughout the R7 subfamily, a
 481 hypothesis which is supported both by the solving of the crystal structure for RGS7, also in
 482 complex with $G\beta_5$ (Patil et al., 2018), but also through structure homology modeling of RGS6L α 2
 483 (**Fig. 9A**, inset). Therefore, loss of $G\beta_5$ expression with RGS6 removal would indicate that
 484 RGS6 is the only R7 family member present in that tissue.

485 These analyses demonstrated that, while most tissues expressing RGS6 also express
 486 other R7 family members ($G\beta_5$ expression was maintained after RGS6 loss), there were some

487 tissues, including: lung, bladder, stomach, intestine, and omental fat, where RGS6 appeared to
488 be the only R7 family member present ($G\beta_5$ expression was completely lost with loss of RGS6,
489 **Fig. 9A**). Indeed, western blot analysis of a subset of the tissues showing a concomitant loss of
490 RGS6 and $G\beta_5$ expression (lung, omental fat, and bladder) using antibodies against individual
491 R7 family members (RGS's 7, 9-2, and 11) confirmed that RGS6 is the only R7 member
492 expressed there (**Fig. 9B**).
493

494 **Discussion**

495 RGS6 pre-mRNA is subject to alternative splicing producing 36 unique transcripts each
 496 predicted to produce viable protein isoforms (Chatterjee et al., 2003). Despite knowing about
 497 this complex processing, sequence similarities have complicated the study of individual RGS6
 498 isoforms. As such, most research implicating RGS6 in neuropsychiatric disorders or exploring
 499 its diverse peripheral roles have taken a global view of its function. This work provides the first
 500 comprehensive analysis of RGS6 isoform expression. Using three distinct antibodies generated
 501 against the whole RGS6L protein, the N-terminus of RGS6L isoforms, and an alternative amino
 502 acid sequence found in the C-terminus of 56% of RGS6 isoforms, we have not only begun to
 503 define tissue-specific differences in RGS6 isoform expression but have also identified brain-
 504 specific RGS6 protein bands that appear to be phosphorylated/dephosphorylated only in CNS
 505 tissues.

506
 507 **RGS6L(+GGL) isoforms are the most prevalent isoforms expressed *in vivo***

508 Western blot analyses demonstrate that RGS6L(+GGL) isoforms predominate throughout the
 509 mouse body (**Fig. 5**, magenta arrow). As the nine possible RGS6L(+GGL) isoforms fall within a
 510 narrow molecular weight range (53-57kDa, **Fig. 4-1**) we were unable to definitively say which of
 511 these isoforms were present. However, using the RGS6-18 antibody, we conclude that, in the
 512 brain, at least some of the RGS6L(+GGL) isoforms include the amino acid sequence encoded
 513 by exon 18 (**Fig. 4A**, right panel). Furthermore, we hypothesize that RGS6L α 1 and RGS6L α 2,
 514 which differ only in the inclusion and exclusion, respectively, of the amino acid sequence
 515 encoded by exon 18 (**Fig. 3**), may represent the predominant brain RGS6L(+GGL) isoforms.
 516 This hypothesis is supported by the fact that RGS6L α 2 (GenbankTM AF073920) and RGS6L α 1
 517 (GenbankTM AF107619) were the first RGS6L splice forms identified. These results suggest
 518 that RGS6L α 1 and RGS6L α 2 are expressed at relatively high levels compared to other

519 RGS6L(+GGL) transcripts which were not identified until our subsequent cloning study
 520 (Chatterjee et al., 2003, Snow et al., 1999).

521 While the studies above suggest certain RGS6L(+GGL) isoforms may predominate due
 522 to relatively high mRNA transcript expression, this is likely not the only explanation for their
 523 prevalence. The R7 RGS protein subfamily is distinguished by two unique domains outside of
 524 the RGS domain: the DEP/DHEX domain and the GGL domain. Importantly, the GGL domain is
 525 required for interaction with G β_5 , an atypical G β subunit that forms an obligate dimer with all R7
 526 family members, promoting their stability (Chen et al., 2003). Consequently, RGS6L(+GGL)
 527 isoforms may be expected to be more stable (**Fig. 4D**) and thus expressed at higher levels
 528 relative to the RGS6L(-GGL) isoforms, which we previously showed do not bind G β_5 (Liu and
 529 Fisher, 2004).

530 Interestingly, as with the RGS6L(-GGL) isoforms, we did not identify RGS6 protein
 531 bands in the brain corresponding in size to RGS6S isoforms (**Fig. 4A, Fig. 4-1**), despite the fact
 532 their corresponding transcripts were originally cloned from a human brain cDNA library
 533 (Chatterjee et al., 2003). While this may be explained by low transcript abundance, it may also
 534 be caused by protein instability as suggested by our cycloheximide chase experiments (**Fig. 4C**
 535 and **D**). RGS6L and RGS6S protein isoforms arise via the use of alternative transcription start
 536 sites within the *RGS6* gene sequence. Specially, while RGS6L protein isoforms are generated
 537 using a transcription start site upstream of exon 1 of the *RGS6* gene, RGS6S isoforms arise via
 538 a transcription start site within intron 7. Therefore, RGS6S protein isoforms lack the first 140
 539 amino acids present in the N-terminus of RGS6L isoforms, and therefore also lack the
 540 DEP/DHEX domain (**Fig. 3A**). The DEP/DHEX domain is known to promote membrane
 541 targeting of R7 family members by facilitating their interaction with two membrane proteins, the
 542 retina-specific RGS9 anchor protein (R9AP) and its close relative, R7 family binding protein
 543 (R7BP) which is ubiquitously expressed (**Fig. 3A**) (Hu and Wensel, 2002, Lishko et al., 2002,
 544 Martemyanov et al., 2003, Martemyanov et al., 2005, Drenan et al., 2005, Anderson et al., 2007,

Patil et al., 2018). However, the DEP/DHEX domain may also facilitate the interaction between $G\beta_5$ and R7 family members (Cheever et al., 2008). Recent evidence supports a model whereby R9AP/R7BP strengthens the interaction between the DEP/DHEX and GGL domains with $G\beta_5$ promoting R7 RGS protein stabilization (Masuho et al., 2011, Patil et al., 2018). These findings are consistent with our cycloheximide chase experiments demonstrating that RGS6L isoforms are more stable than RGS6S isoforms (**Fig. 4D**).

551

552 Identification of novel RGS6 protein bands

We identified three RGS6 protein bands that were larger (61, 65, and 69kDa) than any protein isoform predicted from our initial cloning effort (≤ 57 kDa, Chatterjee et al., 2003). All three bands were RGS6L isoforms as they were recognized by the RGS6-L antibody (**Fig. 4A and 5**). Of these, the 65 and 69kDa bands appear to be brain-specific (**Fig. 5**) phospho- and dephospho forms of RGS6L, respectively. The 65kDa band, whose expression is reduced in the presence of λ phosphatase and increased by a Ca^{2+} -stimulated kinase activity, represents the first positively identified RGS6 phospho-protein (**Figs. 7B-E**). In contrast, the 69kDa RGS6L protein band is increased by λ phosphatase (**Fig. 7B**) and decreased by Ca^{2+} -stimulated kinase activity (**Fig. 7E**). Both the 61kDa and 69kDa RGS6 isoforms are much larger than predicted from any transcripts identified in our original cloning effort (Chatterjee et al., 2003), suggesting they may represent novel RGS6 isoforms. In support of this hypothesis, northern blot analyses conducted in human brain and peripheral tissues indicate that there is at least one transcript predominantly expressed in the CNS that is larger than the other RGS6 transcripts (which correspond in size to the RGS6L and RGS6S splice-forms) (Snow et al., 1999).

Of the novel RGS6 protein bands described above, the 69kDa RGS6B isoform is of particular interest. Importantly, we have shown that RGS6-specific inhibition of $G\alpha_{i/o}$ modulates several CNS disorders for which RGS6 may be a novel therapeutic target. Remarkably, RGS6^{-/-} mice have reduced anxiety/depression (Stewart et al., 2014), exhibit diminished alcohol

571 seeking/reward (Stewart et al., 2015), and develop Parkinson's disease (Bifsha et al., 2014, Luo
572 et al., 2019) (**Fig. 1**). Re-examination of the western blot data presented in these papers
573 revealed that RGS6B is highly expressed in the brain regions affected by the CNS disorders
574 described above, suggesting it plays a critical role in CNS pathology. Therefore, future studies
575 should focus on identifying the transcript encoding RGS6B so that its function may be further
576 explored. There are several pieces of evidence derived from this study that may aid in
577 transcript identification. First, RGS6B is recognized by the RGS6-L antibody (**Fig. 4A**, middle
578 panel, green arrow), indicating the transcript will contain exons 1-6, which encode the long N-
579 terminus. Second, RGS6B is also recognized by the RGS6-18 antibody (**Fig. 4A**, right panel,
580 green arrow), indicating that the transcript includes exon 18. Finally, RGS6B is present in both
581 mouse and human (**Fig. 8B**), indicating it is highly conserved. Therefore, the sequence of the
582 identified transcript must be derived from highly conserved regions within the *RGS6* gene.

583

584 **Speculating on the possible effects of the rs2332700 SNP on RGS6 expression**

585 In reflecting on the present data and the recent meta-analysis (Consortium, 2019) which linked
586 a SNP (rs2332700) in *RGS6* to autism spectrum disorder, bipolar disorder, major depression,
587 and schizophrenia, it becomes intriguing to speculate on the SNP's functionality. The
588 rs2332700 SNP maps to intron 1 of the *RGS6* gene and is associated with a G>C switch.
589 Interestingly, this SNP is only found in primates (**Fig. 5-2**). While a functional role of this SNP
590 has yet to be established, we hypothesize that it alters RGS6 expression. As RGS6L(+GGL)
591 protein isoforms predominate (**Fig. 5A**, magenta arrow), we hypothesize that it is the
592 physiological functions of these isoforms most highly impacted by the SNP. Interestingly, the
593 RGS6L(+GGL) isoforms predominate not only in the CNS, but also in the periphery (**Fig. 5**),
594 suggesting that the rs2332700 SNP may not only be linked to the psychological pathology of
595 schizophrenia, autism spectrum disorder, bipolar disorder, and major depression, but also
596 peripheral comorbidities associated with these diseases. In support of this hypothesis, we have

597 demonstrated that global loss of RGS6 not only reduces CNS-associated alcohol seeking and
598 reward behavior, in mice, but also protects against peripheral alcohol-mediated heart, liver, and
599 gastrointestinal tract toxicity (Stewart et al., 2015).

600 Another intriguing piece of data from our current study is the discovery of a 69kDa form
601 of RGS6 that is brain-specific (RGS6B). Although the functional nature of RGS6B is unknown,
602 numerous western blot analyses conducted in this paper and others (Stewart et al., 2014,
603 Stewart et al., 2015, Bifsha et al., 2014, Luo et al., 2019) demonstrate that it is highly expressed
604 in several brain regions associated with numerous psychiatric disorders including, but not
605 limited to, alcohol use disorders, anxiety/depression, and Parkinson's disease. In addition, our
606 current data suggests that RGS6B shares several structural similarities with the other
607 predominant RGS6L(+GGL) isoforms (**Fig. 4A**). Therefore, it is intriguing to speculate that
608 RGS6B expression may also be altered by the rs2332700 SNP. However, as RGS6B is not
609 expressed in the periphery, we may surmise that alteration in its functional role would largely
610 have psychological effects.

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- 822

823

824 **Figure Legends**

825 **Figure 1: RGS6 has numerous neuropsychiatric, physiological, and pathophysiological**
826 **roles**

827 This diagram highlights the many neuropsychiatric, physiological and pathophysiological
828 functions that have been attributed to RGS6 as well as the primary literature describing these
829 functions.

830

831 **Figure 2: The RGS6 protein sequence is highly conserved across the animal kingdom**

832 This figure shows a conservation map of the largest RGS6 isoform we previously identified
833 (Chatterjee et al., 2003) in different mammalian species. This isoform is designated
834 RGS6L α 1(+GGL) as it includes the extended N-terminus found in RGS6 long (RGS6L)
835 isoforms, the GGL domain, as well as the alternative α 1 C-terminal sequence encoded by exons
836 18 and α . Each column within the conservation map represents a single amino acid and
837 conservation of color within a column represents amino acid conservation. When looking at the
838 histogram around the outside of the conservation map, green represents 100% amino acid
839 conservation whereas grey represents <100% conservation.

840

841 **Figure 3: Predicted protein structure and binding partners for human RGS6 protein**
842 **isoforms**

843 RGS6 is a member of the R7 subfamily (RGS's 6, 7, 9, 11) distinguished by two additional
844 domains, DEP and GGL, which target these proteins to the membrane (through interaction with
845 R9AP and/or R7BP) and promote their stability (through interaction with G β ₅), respectively. In
846 addition to DEP- and GGL-mediated spatial function and regulation, RGS6's cellular roles are

also likely affected by mRNA splicing and alternative domain inclusion/exclusion. **A)** Illustration of the 36 RGS6 protein isoforms encoded by RGS6 mRNA splice variants we identified (Chatterjee et al., 2003). RGS6 proteins possess either long (RGS6L) or short (RGS6S) N-terminal domains, an incomplete or intact GGL domain, and 9 alternative C-terminal sequences. **B)** Sequence alignment of the N-terminal region of RGS6 to that of RGS7, RGS9 and RGS11. The grey box represents a region of low sequence conservation that was used to develop the RGS6L antibody, RGS6-L. **C)** The protein sequence of an alternatively spliced region of RGS6 present in the C-terminus of 56% of RGS6 protein isoforms that was used to develop RGS6-18 antibody. The approximate location at which the RGS6-L and RGS6-18 antibodies bind to RGS6 proteins is denoted in **(A)** by an antibody icon.

Figure 4: RGS6 antibodies confirm *in vivo* RGS6L expression and identify novel RGS6 protein bands

A) Western blots of whole brain lysates derived from RGS6^{+/+} (WT) and RGS6^{-/-} (KO) mice using antibodies against: the whole RGS6L protein (RGS6-fl, left panel), the N-terminus of RGS6L protein isoforms (RGS6-L, middle panel), and an 18 amino acid sequence found in 56% of RGS6 protein isoforms (RGS6-18, right panel). Magenta arrows represent RGS6 protein bands corresponding in size to RGS6L(+GGL isoforms) (**Fig. 4-1**). Blue and green arrows represent RGS6 protein bands that are larger (61 and 69kDa) than the predicted sizes (≤ 57 kDa) of the RGS6 species we previously identified (Chatterjee et al., 2003). Asterisks represents the RGS7 protein band which is non-specifically recognized by the RGS6-fl and RGS6-18 antibodies. **B)** Western blot analysis demonstrating that while the RGS6-fl antibody recognizes both RGS6L and RGS6S isoforms overexpressed in HEK cells, the RGS6-L antibody specifically recognizes the RGS6L isoforms. Yellow arrows represent additional RGS6 protein bands produced by overexpression of the RGS6L(+/-GGL) and RGS6S(+/-GGL) vector

872 constructs. These bands are smaller than the predicted sizes for the RGS6L(+/-GGL) and
 873 RGS6S(+/-GGL) isoforms and likely represent additional protein isoforms produced by novel
 874 translation start sites encoded by all constructs (**Fig. 4-2**). **C)** Representative western blots
 875 from cycloheximide chase experiments characterizing the differential stability of the RGS6L(+/-
 876 GGL) and RGS6S(+/-GGL) protein isoforms over a 24h period. Yellow arrows represent the
 877 additional low molecular weight RGS6 protein bands recognized by the RGS6-fl antibody in
 878 panel (**B**). These bands were not included in the final analysis. **D)** Densitometric analysis of
 879 protein degradation comparing the four RGS6 protein isoforms ($N = 3$) in (**C**).

880

881 **Figure 4-1: Predicted molecular weights of known RGS6 isoforms**

882 This table may be used as a reference for determining the putative identities of RGS6 protein
 883 bands present on western blot.

884

885 **Figure 4-2: Novel putative translation start sites identified within RGS6 protein sequence**

886 Predicted RGS6L α 1 protein sequence. Red star denotes alternative start site utilized to
 887 produce RGS6S isoforms. Yellow stars denote novel putative translation start sites present in
 888 both RGS6L and RGS6S isoforms.

889

890 **Figure 5: Tissue expression profiling of RGS6 in 31 mouse tissues using the RGS6-L** 891 **antibody**

892 Using purified RGS6-L antibody we conducted an analysis of RGS6L expression in 31 tissues
 893 throughout the mouse body. **A)** Representative western blots demonstrating that, in RGS6^{+/+}
 894 (WT) and RGS6^{+/-} (H) mice compared to RGS6^{-/-} (KO) mice, many CNS and peripheral tissues

895 express the 53-57kDa RGS6L(+GGL) protein isoforms (magenta arrow) identified previously
 896 (Chatterjee et al., 2003). The blue and green arrows indicate the novel 61 and 69kDa RGS6
 897 protein bands identified in **Fig. 4A**, and the orange arrow indicates a third novel 65kDa band
 898 identified in the larger expression profile. While the 65 and 69kDa RGS6 protein bands were
 899 brain-specific, the 61kDa band was identified in multiple tissues. **Note:** this figure was
 900 constructed from multiple gels. Therefore, we have placed vertical black lines between tissue
 901 samples to denote RGS6 expression levels cannot be directly compared between tissues. **B)**
 902 Densitometric quantitative analysis of the 53-57kDa RGS6L(+GGL) isoforms (**Fig 5A**, magenta
 903 arrow) reveal that they are significantly expressed in CNS tissues as well as peripherally in
 904 atrium and bladder. Two-way ANOVA found significant effects of strain ($F_{2,258} = 143.75$, $P \leq$
 905 0.0001), tissue ($F_{30,258} = 44.11$, $P \leq 0.0001$), and their interaction ($F_{59,258} = 15.93$, $P \leq 0.0001$).
 906 Data were analyzed via two-way ANOVA with Fisher's LSD post hoc adjustment. Data are
 907 presented as mean $\pm$ S.E.M. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$. ($N = 3-8$
 908 RGS6^{+/+} mice, 2-6 RGS6^{+/-} mice, and 2-8 RGS6^{-/-} mice). Extended data in **Fig. 5-1** compares
 909 significant differences in RGS6 expression across tissues in RGS6^{+/+} mice. **Fig. 5-2** shows the
 910 conservation of an RGS6 SNP in primates. **Abbreviations:** CTX = cerebral cortex, OFB =
 911 olfactory bulb, MB = midbrain, CB = cerebellum, SC = spinal cord, Ret = retina, WAT = white
 912 adipose tissue, BAT = brown adipose tissue, Ofat = Omental fat, Spl = spleen, Thy = thymus,
 913 Pros = prostate, Test = testis, Brst = breast, Ov = ovary, Ut = uterus, Atr = atrium, Vent =
 914 ventricle, Dia = diaphragm, Sk mus = skeletal muscle, Eso = esophagus, Stom = stomach, S int
 915 = small intestine, L int = large intestine, Panc = pancreas, Kid = kidney, Ad gl = adrenal gland,
 916 Blad = bladder, Plat = platelet.

917
 918 **Figure 5-1: Comparison of RGS6 expression across 31 mouse tissues using the RGS6-L**
 919 **antibody.**

920 Using the RGS6^{+/+} (WT) tissue data from **Figure 5**, we have graphed RGS6 protein expression
921 detected with the RGS6-L antibody across the same 31 tissues. Here, we find that RGS6 is
922 most highly expressed in the cerebral cortex (CTX, $P \leq 0.000$) relative to all other tissues tested.
923 Data are represented as mean $\pm$ S.E.M. Data were analyzed via one-way ANOVA with Fisher's
924 LSD *post-hoc* adjustment. Table below displays the *P* values of all comparisons of RGS6
925 expression across the various tissues. **Abbreviations:** CTX = cerebral cortex, OFB = olfactory
926 bulb, MB = midbrain, CB = cerebellum, SC = spinal cord, Ret = retina, WAT = white adipose
927 tissue, BAT = brown adipose tissue, Ofat = Omental fat, Spl = spleen, Thy = thymus, Pros =
928 prostate, Test = testis, Brst = breast, Ov = ovary, Ut = uterus, Atr = atrium, Vent = ventricle, Dia
929 = diaphragm, Sk mus = skeletal muscle, Eso = esophagus, Stom = stomach, S int = small
930 intestine, L int = large intestine, Panc = pancreas, Kid = kidney, Ad gl = adrenal gland, Blad =
931 bladder, Plat = platelet.

932

933 **Figure 5-2: Evolutionary conservation of the rs2332700 SNP.**

934 Human RGS6 sequence (37bps) flanking the rs2332700 SNP (G>C) aligned against the
935 corresponding sequence present in 30 different animal species across the animal kingdom
936 using the UCSC Genome Browser. The SNP is only found in primates. "=": gap regions in
937 genomes, in which aligning species has one or more unalignable bases. This is likely due to
938 excessive evolutionary distance between the aligning species and human or due to independent
939 insertions/deletions in the region between the aligning species and human.

940

941 **Figure 6: Immunohistochemical analysis of RGS6 tissue distribution**

942 Representative images from an immunohistochemical analysis of RGS6 protein distribution,
943 using the RGS6-fl antibody, in mouse RGS6^{+/+} (WT) CNS (**Left panel**) and peripheral (**Right**

944 **panel)** tissues shown to express RGS6 protein bands via western (**Fig. 5A**). Complimentary
945 tissues collected from RGS6^{-/-} (KO) controls confirmed that the RGS6-fl antibody, while it
946 recognizes RGS7 via western blots (**Fig. 4A**), is specific for RGS6 in tissue sections.

947

948 **Figure 7: Prediction of RGS6Lα1 phosphorylation sites and identification of a novel,**
949 **~65kDa brain-specific RGS6 phosphoprotein**

950 **A)** Putative phosphorylation sites within the RGS6Lα1 protein were identified using the GPS 3.0
951 software (**Fig. 7-1**). Sites with the highest prediction score were mapped to the RGS6Lα1
952 protein sequence with the corresponding kinase labeled. Serine residues are indicated in blue,
953 threonine in pink, and tyrosine in green. **B)** A representative western blot from RGS6^{+/+} (WT)
954 and RGS6^{-/-} (KO) brain tissue lysates treated where indicated with λ phosphatase. The
955 magenta arrow indicates the ~53-57kDa RGS6L(+GGL) isoforms, while the blue, orange, and
956 green arrows indicate the novel RGS6 protein bands (61, 65, and 69kDa, respectively) identified
957 in this study. **C)** A densitometric quantification of the 56kDa, 61kDa, 65kDa, and 69kDa RGS6
958 protein bands (**B**). Two-way ANOVA with Fisher's LSD *post-hoc* adjustment revealed a
959 significant effect of λ phosphatase on the expression of the 65kDa ($P \leq 0.0001$) and 69kDa ($P \leq$
960 0.0001) bands relative to their respective untreated samples. Data are presented as mean ±
961 S.E.M. **** $P \leq 0.0001$. ($N = 3$ mice per treatment). **D)** A representative western blot from
962 RGS6^{+/+} (WT) and RGS6^{-/-} (KO) brain tissue lysates treated where indicated with PMA (200nM),
963 cAMP and Forskolin (10μM of each), or Ca²⁺(10μM). **E)** A densitometric quantification of the
964 56kDa, 61kDa, 65kDa, and 69kDa RGS6 protein bands (**D**). Two-way ANOVA with Fisher's
965 LSD *post-hoc* adjustment found a significant effect of Ca²⁺ treatment on the expression of the
966 65kDa ($P \leq 0.0001$) and 69kDa ($P = 0.002$) bands relative to their respective untreated samples.

967

968 **Figure 7-1: Putative phosphorylation sites in the RGS6Lα1 protein**

969 X denotes sites that were also predicted by PhosphoNet or have been confirmed as
970 phosphorylated residues through mass spectrometry data sets.

971

972 **Figure 8: The 69kDa brain-specific RGS6 protein band is highly conserved and**
973 **significantly expressed in all mouse CNS tissues analyzed**

974 **A)** Densitometric quantification of the 69kDa, brain-specific RGS6 protein band (**Fig. 5A**, green
975 arrow) demonstrates that it is significantly expressed in all RGS6^{+/+} (WT) mouse brain tissues,
976 compared to RGS6^{+/-} (H) and RGS6^{-/-} (KO) tissues analyzed. Two-way ANOVA found
977 significant effects of strain ($F_{2,32} = 99.51$, $P \leq 0.0001$), tissue ($F_{4,32} = 21.19$, $P \leq 0.0001$), and
978 their interaction ($F_{8,32} = 21.19$, $P = 0.000$). Data were analyzed via two-way ANOVA with
979 Fisher's LSD post hoc adjustment. Data are presented as mean $\pm$ S.E.M. * $P \leq 0.05$, ** $P \leq 0.01$,
980 *** $P \leq 0.001$, **** $P \leq 0.0001$. ($N = 3-4$ RGS6^{+/+} mice, 2-3 RGS6^{+/-} mice, and 3-4 RGS6^{-/-} mice).
981 Extended data in **Fig. 8-1** compares significant differences in RGS6B expression across tissues
982 in RGS6^{+/+} mice. **Abbreviations:** CTX = cerebral cortex, CB = cerebellum, MB = midbrain, SC
983 = spinal cord, OFB = olfactory bulb. **B)** Western blot analysis of mouse and human brain
984 lysates indicate that the 61 and 69kDa, brain-specific protein bands (blue and green arrows,
985 respectively) are conserved in mouse and human.

986

987 **Figure 8-1: Comparison of RGS6B expression across CNS mouse tissues using the**
988 **RGS6-L antibody.**

989 Using the RGS6^{+/+} (WT) tissue data from **Fig. 8**, we have graphed RGS6B protein expression
990 detected with the RGS6-L antibody across mouse CNS tissues. Data are represented as mean

991 $\pm$ S.E.M. Data were analyzed via one-way ANOVA with Fisher's LSD *post-hoc* adjustment.
 992 Table below displays the *P* values of all comparisons of RGS6 expression across the various
 993 tissues. **Abbreviations:** CTX = cerebral cortex, CB = cerebellum, MB = midbrain, SC = spinal
 994 cord, OFB = olfactory bulb.

995

996 **Figure 9: RGS6 is the only R7 family member expressed in bladder, lung, omental fat,**
 997 **stomach, and intestine**

998 **A)** Densitometric quantification of G β_5 expression in tissues shown to express RGS6 protein
 999 bands via western (**Fig. 5A**). **Inset:** Structure homology modeling of RGS6L α 2 in complex with
 1000 G β_5 . Two-way ANOVA found significant effects of strain ($F_{2,135} = 19.69$, $P \leq 0.0001$) and tissue
 1001 ($F_{15,135} = 90.64$, $P \leq 0.0001$). Data were analyzed via two-way ANOVA with Fisher's LSD post
 1002 hoc adjustment. Data are presented as mean $\pm$ S.E.M. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$,
 1003 **** $P \leq 0.0001$. ($N = 2-6$ RGS6 $^{+/+}$ mice, 2-5 RGS6 $^{+/-}$ mice, and 3-6 RGS6 $^{-/-}$ mice). Extended
 1004 data in **Fig. 9-1** compares significant differences in G β_5 expression across tissues in RGS6 $^{+/+}$
 1005 mice. **Abbreviations:** CTX = cerebral cortex, CB = cerebellum, MB = midbrain, SC = spinal
 1006 cord, OFB = olfactory bulb, Ret = retina, Ofat = Omental fat, Atr = atrium, Vent = ventricle, Brst
 1007 = breast, Stom = stomach, S int = small intestine, L int = large intestine, Kid = kidney, Blad =
 1008 bladder. **B)** Representative western blot analysis of a subset of the tissues from (**A**) showing a
 1009 concomitant loss of RGS6 and G β_5 expression using antibodies against the individual R7 family
 1010 members confirmed that RGS6 is the only R7 member expressed there. **Note:** The G β_5 portion
 1011 of this figure was constructed from multiple blots. Therefore, we have placed vertical black lines
 1012 between G β_5 tissue samples to denote expression levels cannot be directly compared. The first
 1013 lane labeled "WT" is a lysate from whole brain for all blots except for the blot for RGS9-2, where
 1014 it is striatum, as RGS9-2 is not detectable in whole brain lysates (Luo et al., 2019).
 1015 **Abbreviations:** WT = RGS6 $^{+/+}$, H = RGS6 $^{+/-}$, and KO = RGS6 $^{-/-}$.

1016

1017 **Figure 9-1: Comparison of Gβ₅ expression across 16 mouse tissues.**

1018 Using the RGS6^{+/+} (WT) tissue data from **Fig. 9**, we have graphed Gβ5 protein expression
 1019 across 16 mouse tissues. Data are represented as mean ± S.E.M. Data were analyzed via
 1020 one-way ANOVA with Fisher's LSD *post-hoc* adjustment. Table below displays the *P* values of
 1021 all comparisons of RGS6 expression across the various tissues. **Abbreviations:** CTX =
 1022 cerebral cortex, CB = cerebellum, MB = midbrain, SC = spinal cord, OFB = olfactory bulb, Ret =
 1023 retina, Ofat = Omental fat, Atr = atrium, Vent = ventricle, Brst = breast, Stom = stomach, S int =
 1024 small intestine, L int = large intestine, Kid = kidney, Blad = bladder.

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A

B

RGS6-L Antibody

RGS6 : MAQSGDQRAVGADPEESSPNMIVYCKIED...
RGS7 : MAQGNN--YGQTSNGVADESPNMLVYRKMED...
RGS9 : MTI--R-----HQGQYRPRMAFLQKIEA...
RGS11: MAAGPA-----PPPGRPRAQMPHLRKMER...

*: . * *: *

C

RGS6-18 Antibody

RGS6 : PESEQGRRTSLEKFTRSV...