Title
Association between regulator of G protein signaling 9-2 and body weight.

Permalink
https://escholarship.org/uc/item/8h01013r

Journal
PloS one, 6(11)

ISSN
1932-6203

Authors
Waugh, Jeffrey L
Celver, Jeremy
Sharma, Meenakshi
et al.

Publication Date
2011

DOI
10.1371/journal.pone.0027984

Peer reviewed
Association between Regulator of G Protein Signaling 9–2 and Body Weight

Jeffrey L. Waugh1*, Jeremy Celver2,3*, Meenakshi Sharma2, Robert L. Dufresne4, Dimitra Terzi5, S. Craig Risch6, William G. Fairbrother7, Rachael L. Neve8, John P. Kane9,10,11, Mary J. Malloy9, Clive R. Pullinger9,12, Harvest F. Gu13, Christos Tsatsanis5, Steven P. Hamilton6, Stephen J. Gold1, Venetia Zachariou2, Abraham Kovoor2,3.

1 Department of Psychiatry, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, United States of America, 2 Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, Rhode Island, United States of America, 3 Kovogen LLC, Mystic, Connecticut, United States of America, 4 Department of Pharmacy Practice, University of Rhode Island, Kingston, Rhode Island, United States of America, 5 Department of Basic Sciences, Faculty of Medicine, University of Crete, Heraklion, Crete, Greece, 6 Department of Psychiatry, University of California San Francisco, San Francisco, California, United States of America, 7 Department of Molecular and Cell Biology and Biochemistry, Brown University, Providence, Rhode Island, United States of America, 8 Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts, United States of America, 9 Cardiovascular Research Institute, University of California San Francisco, San Francisco, California, United States of America, 10 Department of Medicine, University of California San Francisco, San Francisco, California, United States of America, 11 Department of Biochemistry and Biophysics, University of California San Francisco, San Francisco, California, United States of America, 12 Department of Physiological Nursing, University of California San Francisco, San Francisco, California, United States of America, 13 Department of Molecular Medicine and Surgery, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden

Abstract

Regulator of G protein signaling 9–2 (RGS9–2) is a protein that is highly enriched in the striatum, a brain region that mediates motivation, movement and reward responses. We identified a naturally occurring 5 nucleotide deletion polymorphism in the human RG9 gene and found that the mean body mass index (BMI) of individuals with the deletion was significantly different from those without. A splicing reporter minigene assay demonstrated that the deletion had the potential to significantly decrease the levels of correctly spliced RG9 gene product. We measured the weights of rats after virally transduced overexpression of RG9–2 or the structurally related RGS proteins, RG57, or RG511, in the nucleus accumbens (NAc) and observed a reduction in body weight after overexpression of RG9–2 but not RG57 or 11. Conversely, we found that the RG9 knockout mice were heavier than their wild-type littermates and had significantly higher percentages of abdominal fat. The constituent adipocytes were found to have a mean cross-sectional area that was more than double that of corresponding cells from wild-type mice. However, food intake and locomotion were not significantly different between the two strains. These studies with humans, rats and mice implicate RGS9–2 as a factor in regulating body weight.

Citation: Waugh JL, Celver J, Sharma M, Dufresne RL, Terzi D, et al. (2011) Association between Regulator of G Protein Signaling 9–2 and Body Weight. PLoS ONE 6(11): e27984. doi:10.1371/journal.pone.0027984

Editor: Malu G. Tansey, Emory University, United States of America

Received September 1, 2011; Accepted October 28, 2011; Published November 23, 2011

Copyright: © 2011 Waugh et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The project described was supported by an R41MH78570 award from the National Institute of Mental Health (NIMH), NIH (to AK, JC and SPH), and a Rhode Island IDeA Network of Biomedical Research Excellence (RI-INBRE) Award, P20RR016457-10, from the National Center for Research Resources (NCRR), NIH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have read the journal's policy and have the following conflicts: Jeremy Celver is an employee of, and Abraham Kovoor is an owner of the biotechnology company, Kovogen, LLC. However, the two missions of Kovogen LLC are the following: 1) Develop biomarkers for risk susceptibility to neurological side-effects of antipsychotic drugs. 2) Develop molecular probes for Magnetic Resonance Imaging of specific biomolecules. Therefore, neither Jeremy Celver, Abraham Kovoor nor Kovogen, LLC, has any commercial interest in the results described in the manuscript and have not applied for any patent that relates to these results. The affiliations to Kovogen, LLC, do not alter the authors’ adherence to all the PLoS ONE policies on sharing data and materials.

* E-mail: abekovoor@uri.edu

* These authors contributed equally to this work.

Introduction

A large body of data indicates that brain circuits in the striatum that utilize opioid peptides and dopamine as neurotransmitters are important in i) the motivation to acquire food, ii) encoding food value and reward and iii) the orchestration of movements for acquiring food [1,2].

Regulators of G-protein signaling (RGS) are a family of proteins that can accelerate GTP hydrolysis catalyzed by G protein coupled receptor (GPCR)-activated, Gα G protein subunits. Consequently, they accelerate the termination of GPCR signals [3]. In addition to the class-defining RGS domain which is responsible for the GTPase accelerating function (GAP) function, RGS proteins also contain additional regions that mediate intracellular interactions and non-canonical functions that are distinct from the canonical GAP function [4].

This study which investigates the role of the striatally enriched RGS protein, RGS9–2 [5], in regulating body weight was prompted by the following findings. First, RGS9–2 specifically modulates the GPCRs, D2-like dopamine receptors (D2R) [6,7,8,9], and mu opioid receptors [10,11,12]. Second, D2R and mu opioid receptor signaling in the striatum regulates feeding...
behavior, body weight [1,2], and reward responses [13,14]. Third, several studies have shown that altered RGS9−2 levels modulate the reward responses to drugs that activate brain opioid and dopamine receptors [15].

RGS9−1 and RGS9−2 are the short and long splice variants, respectively, of the RGS9 gene and the expression of each of these variants is highly tissue specific. RGS9−2 is expressed specifically in the brain and is highly enriched in striatum neurons while RGS9−1 is thought to be expressed specifically in the retina [5,16,17]. RGS9−1 and 2 are members of the R7 RGS protein subfamily [18] whose members are defined by the presence of two N-terminal domains: i) a DEP (for dishevelled/EGL-10/pleckstrin homology) domain and ii) GY-like domain (GGG) that binds Gβ5, an outlying member of the G protein beta subunit family.

Here, we identify a polymorphism in the human RGS9 gene likely to alter functional levels of RGS9−2. Based on our observations that RGS9 knockout mice were heavier than their wild-type littermates we hypothesized that humans with the ΔTTTCT polymorphism were on average heavier than those who were negative for the polymorphism.

A preliminary screen indicated that the ΔTTTCT deletion polymorphism was rare in Caucasians and African Americans, occurring at a frequency of less than 1%. As a result of the infrequency of the deletion in these ethnic groups we estimated that we would not be able identify enough ΔTTTCT deletion positive Caucasians or African Americans to test for an association between the ΔTTTCT polymorphism and body weight.

However, our preliminary screen suggested that ΔTTTCT was more common in East Asians occurring at significantly higher frequencies. Therefore this ethnic group was studied to test for a relationship between the ΔTTTCT deletion and body weight. We genotyped 491 individuals of Chinese, Filipino, Japanese, Korean, Pacific Islander, or Southeast Asian ancestry from whom blood samples had previously been obtained along with height, weight, age and sex data.

Forty nine individuals were homozygous for the ΔTTTCT deletion (ΔTTTCT+/+), 197 individuals were heterozygous for the deletion (ΔTTTCT+/−), and 245 individuals tested negative for the deletion (ΔTTTCT−/−). The mean body mass index (BMI) was 25 (SD = 4.5) for the group homozygous for the deletion and 24.9 (SD = 4.4) for individuals heterozygous for the deletion. The mean BMI of the 245 individuals that tested negative for the deletion (ΔTTTCT−/−) was 24.2 (SD = 4.1) and was significantly different (t = 1.99, p<0.05) from the mean BMI, 24.9 (SD = 4.4), of the 246 individuals that tested positive for the deletion (ΔTTTCT+/−, i.e. individuals either homozygous, ΔTTTCT+/+, or heterozygous, ΔTTTCT+/−, for the deletion).

Table 1 outlines the ethnic sub-composition of the East Asian population sample, the frequency of the occurrence of the deletion in each sub-group and the mean BMIs of the ΔTTTCT+/+ and

| Ethnicity                           | ΔTTTCT+/+ | ΔTTTCT+/− | ΔTTTCT−/− |
|------------------------------------|-----------|-----------|-----------|
| Chinese (45.0%)                     | 24.4±4.6  | 23.2±3.5  | 23.8±4.1  |
| Japanese (15.9%)                    | 25.1±5.6  | 24.4±4.2  | 24.6±4.8  |
| Korean (4.7%)                       | 24.3±3.4  | 23.8±4.1  | 23.9±4.0  |
| Pacific Islander (21.0%)            | 25.9±3.9  | 25.7±4.2  | 25.8±4.0  |
| Southeast Asian (13.4%)             | 25.1±3.3  | 25.3±5.0  | 25.2±4.1  |

*Table 1. Table outlining the body mass index (BMI) associated with each ethnic group and with ΔTTTCT deletion positive (Δ+) and ΔTTTCT deletion negative groups.*

Table Key:
ΔTTTCT deletion positive: Δ−: ΔTTTCT deletion negative
*Percentages in parenthesis in the first column indicate percent fraction of the study population that belong to each ethnic group.
Numbers in parenthesis in columns 2–4 indicate the number of subjects associated with each cell.
Ethnic groups have significantly different BMI’s (ANOVA, F = 471, p<0.005); follow-up Fisher’s Least Significant Difference test (p<0.05) test shows Chinese have lower BMI than Southeast Asians or Pacific Islanders and Koreans having a lower BMI than Pacific Islanders.
Student independent t-test shows significant difference (p<0.05) between ΔTTTCT deletion positive (Δ+) and ΔTTTCT deletion negative (Δ−) individuals.

Table Key:
ΔTTTCT deletion positive: Δ−: ΔTTTCT deletion negative
*Percentages in parenthesis in the first column indicate percent fraction of the study population that belong to each ethnic group.
Numbers in parenthesis in columns 2–4 indicate the number of subjects associated with each cell.
Ethnic groups have significantly different BMI’s (ANOVA, F = 471, p<0.005); follow-up Fisher’s Least Significant Difference test (p<0.05) test shows Chinese have lower BMI than Southeast Asians or Pacific Islanders and Koreans having a lower BMI than Pacific Islanders.
Student independent t-test shows significant difference (p<0.05) between ΔTTTCT deletion positive (Δ+) and ΔTTTCT deletion negative (Δ−) individuals.
ΔTTTCT-ve individuals in each group. The frequency of the incidence of the deletion was significantly different ($\chi^2 = 16$, $p<0.005$) between some of the different ethnic groups examined; those having Japanese and Korean ancestry had the lowest frequency of occurrence of ΔTTTCT.

Analysis of variance of the relationship of ethnicity among the East Asian subjects on body mass index shows significant differences between those identifying themselves as Chinese, Japanese, Korean, Pacific Islander, or Southeast Asian. The different East Asian ethnic groups have significantly different BMIs (ANOVA, $P<0.005$); follow-up Fisher’s Least Significant Difference test ($P<0.05$) test shows that Chinese have lower BMI than Southeast Asians or Pacific Islanders and Koreans have a lower BMI than Pacific Islanders. However, we found that within each ethnic group, the differences in the BMI between deletion-positive and deletion-negative individuals were not significant.

Men had a significantly higher ($t=6.56$, $p<0.001$) BMI than women ($25.8\pm3.6$ versus $23.4\pm4.5$, Mean±SD, Table 2), but, as would be expected by the chromosome 17 location of the RGS9 gene, there was no association ($\chi^2=0.74$) between sex and presence of the deletion and the deletion is similarly distributed in men and women. Therefore, unequal gender distribution between the deletion positive and negative groups does not account for the difference in BMI between the two groups. Finally, there was no relationship between BMI and age in our study population.

A more detailed description of the East Asian study subjects are provided in the Supporting Information Tables S1 and S2.

**Effects of the ΔTTTCT deletion polymorphism on splicing of minigene construct**

Since the ΔTTTCT deletion was close to the branch point for RNA splicing and corresponded to a binding motif for PTB, we attempted to determine if the deletion could alter splicing of the RGS9 gene transcript. Analysis of RNA from the tissue of individuals with the ΔTTTCT polymorphism would be the ideal method of assessing the effect of this deletion on splicing. However, RNA extracted from tissues of ΔTTTCT+ve individuals was not available to us. In addition, the splicing of the RGS9 transcript is highly tissue specific. For example, in humans, the shorter alternatively spliced form, RGS9-1 is highly expressed in the retina but not detectable in the brain, while RGS9-2 is largely expressed in the brain [17] and not detectable in the retina. Therefore it is not certain that the effect of ΔTTTCT on splicing of RGS9 RNA in an accessible tissue such as blood would be reflective of the splicing in the tissues responsible for the increased BMI phenotype. Furthermore, the RGS9 gene transcript is very large (>170 kilobases) [17] and splicing alterations observed in *ciao* for such large transcripts cannot be definitively linked to a particular gene variation without sequencing the entire gene sequence for all the individuals in the study sample. In practice, such extensive sequencing was not possible and therefore we would not definitively exclude the possibility that the altered splicing arose, instead, from alternative undiscovered variations in non-sequenced sections of the gene.

To circumvent such constraints splicing reporter minigene assays are often used to assess the impact of allelic variants on splicing [21,22]. These assays examine the splicing of a minigene reporter constructed using the portion of the gene containing the variation and allow for a more definitive linkage of the gene variation to splicing alterations. As described in Methods and Materials and depicted schematically in Fig. 1A, an RGS9 gene fragment containing exon 13 and intron 13 was utilized to construct a splicing reporter minigene which was transfected into HEK293 cells. The resulting gene transcripts were analyzed by reverse transcriptase PCR (RT-PCR) and gene sequencing. Incorporation of ΔTTTCT deletion into the RGS9 minigene fragment increased the frequency of exon skipping during splicing of the minigene transcript and decreased the fraction of the correctly spliced transcript (i.e. transcript containing all 4 minigene exons, Figs. 1B & C).

**Table 2.** Table outlining the mean body mass index (BMI) associated with males and females and with ΔTTTCT deletion positive (Δ+) and ΔTTTCT deletion negative (Δ-) deletion negative male and female subjects.

| SEX               | Δ- | Δ+ | Δ- & Δ+ | No. of Subjects |
|-------------------|----|----|---------|-----------------|
| Females (51.7%)   | 23.9±4.8 (122) | 22.9±4.0 (132) | 23.4±4.5 (254) | 108 |
| Males (48.3%)     | 26.3±3.7 (124) | 25.6±3.6 (113) | 25.8±3.6 (237) | 148 |
| All Subjects      | 24.9±4.4 (246) | 24.2±4.1 (245) | 24.6±4.2 (491) | 256 |

Table Key:
- Δ-: ΔTTTCT deletion positive; Δ+: ΔTTTCT deletion negative
- Percentages in parenthesis in the first column indicate percent fraction of the study population that is either female or male.
- Numbers in parenthesis in columns 2–4 indicate the number of subjects associated with each cell.
- Men had a significantly higher (Student independent t-test, $t=6.56$, $p<0.001$) BMI than women.
- Student independent t-test shows significant difference (p<0.05) between ΔTTTCT deletion positive (Δ+) and ΔTTTCT deletion negative (Δ-) individuals.

To quantify the development of this phenotype, we recorded the weight of wild-type and homozygous RGS9 knockout (rgs9-/-) mice born from heterozygote breeders that were given *ad libitum* access to water and 4% fat rodent chow. Mice were weighed daily from weaning until post-weaning day 55 (postnatal days 21 through 76).

The weights of both male and female rgs9-/- diverged from their wild-type littermates, at approximately 19 g for females and 21 g for males, and this disparity increased through day 50 post-weaning (Fig. 2B and C). Though we did not systematically measure body weight past day 55, observation of older animals, indicated that the increased weight of RGS9 knockout animals persisted at least through 12 months of age.

A measurement of the food consumed indicated that the altered weight of the knockout mice was not due to increased food intake. The amounts of food consumed by the rgs9-/- and rgs9+/- were similar: 3.22±0.29 and 3.07±0.29 g/day (Mean±SEM) for the wild-type mice and the RGS9 knockout mice, respectively.

The locomotor activity of the wild-type and RGS9 knockout male and female mice measured over a 2 hr time period was similar (Fig. 2D). At an earlier time point (30 min) the male rgs9-/- mice demonstrated a small but significantly increased ambulatory activity when compared to their wild-type counterparts. This small increase in initial activity could represent an increased anxiety phenotype for these mice, but cannot easily explain their increased weight.
Enlarged fat pads and adipocytes in RGS9 knockout mice and undetectable levels of RGS9 protein expression in visceral fat depots of wild-type mice

We dissected visceral white adipose tissue from mesenteric, epididymal and perirenal depots from age-matched wild-type and RGS9 knockout male mice between 2 and 4 months old and found significantly larger amounts of visceral adipose tissue in the RGS9 knockout mice (Fig. 3A).

The epididymal adipose tissue from 8 week old male or female wild-type mice was Western blotted and probed with a validated antibody previously shown to recognize both the long and short RGS9 isoforms [23]. While RGS9-2 protein expression was clearly detected in striatal tissue, we were unable to detect any RGS9 protein signal in the visceral fat from the same mouse using this approach (Fig. 3B). We also further validated the antibody by confirming that no protein signal was detected when Western blots of striatal tissue from the rgs9-/- mice were probed with the antibody (data not shown).

An analysis of the epididymal (Fig. 3C, D and E) or mesenteric depots showed that the adipocytes from RGS9 knockout animals were significantly enlarged when compared to the wild-type animals (Fig. 3D and E).

RGS9-2 over-expression in rat nucleus accumbens (NAc) leads to weight loss

Following our observations of the increased weight of RGS9 knockout mice, we tested whether overexpression of RGS9-2 in the NAc could produce weight loss. We used a previously validated Herpes Simplex Virus (HSV) delivery system to drive overexpression of RGS9-2 in the nucleus accumbens (NAc) shell of male rats [7,24] while quantifying their body weight dynamics. The effects of RGS protein overexpression on body weight were compared with the effects of control LacZ expression.

Rats injected with the HSV RGS9-2 construct exhibited enhanced weight-loss relative to the LacZ controls. The loss in weight of the HSV RGS9-2 injected rats relative LacZ was...
greatest on day 3 after surgery (Fig. 4) and averaged 5.17 g (1.7% of initial body mass).

It has been shown previously that HSV-transduced RGS9-2 protein expression in the rat NAc peaks on day 3–4 post-injection and rapidly declines thereafter [7]. The relative weight loss experienced by the HSV-RGS9-2 injected rats was similarly transient: on post-surgical day 5, the body weights of the RGS9-2 expressing animals were no longer significantly different from the LacZ overexpressing controls. By day 15 following viral injection, group weights were indistinguishable (Fig. 4).

In contrast, overexpression of the structurally similar proteins, RGS7 and RGS11, in rat NAc shell led to weight gain relative to LacZ overexpression (Fig. 4). For example, on day 3 post-surgery, RGS11 overexpressing rats weighed on average 10.8 g (3.6% of total body mass) more than the LacZ controls.

Discussion

In this manuscript we provide data from human, mouse and rat studies that suggest that RGS9-2, a brain specific RGS family member, can regulate body weight and adiposity. First we link an intronic deletion in the RGS9 gene to increased body mass index (BMI). We provide mechanistic insights into this association by showing i) that the intronic deletion is coincident with a binding motif for the polypyrimidine tract binding protein (PTB), a protein involved in regulating the processing of RNA and ii) that the intronic variation can alter the splicing of the RGS9 gene product. Further confirmation for a role of RGS9 in regulating body weight is provided by observations showing that RGS9 knockout mice are heavier than their wild-type littermates, have increased adiposity and adipocytes with approximately doubled cross-sectional area. Finally we identify a possible site for the action of RGS9-2 in regulating body-weight by showing that overexpression of RGS9-2 in the nucleus accumbens of rats can decrease body-weight.

A major finding of our study is that an intronic deletion polymorphism in the RGS9 gene (DTTTCT) is associated with significantly increased BMI in humans. The increase in mean BMI (24.9 versus 24.2) represents an increase in weight from 68.2 kg (150 lb) to 70 kg (154 lb), or a 2.7% increase in weight, for an individual 168 cm (5 ft 6 inch) tall. Thus, the influence of the DTTTCT deletion polymorphism on body weight is quite mild: a World Health Organization panel has suggested that the obesity cut-off definition for Asians should be lowered to a BMI of 25 [25], compared to 35 for Caucasians, but the mean BMI of the DTTTCT deletion positive East Asian individuals in our study is less than even this lowered BMI value.

An examination of Table 1 shows that the increase in mean BMI of the ΔTTTCT deletion positive subjects relative to the deletion negative individuals is greatest among the Chinese, is very small in Pacific Islanders and is reversed in Southeast Asians. However, the above differences and the differences in BMI between deletion positive and negative individuals within each ethnic sub-group were not statistically significant. Nevertheless, it...
is interesting to note that since Pacific Islanders (Polynesians) are descendants of voyagers from Southeast Asia—either Taiwanese aborigines or former inhabitants of New Guinea and the surrounding islands [26,27], they are more closely related genetically to Southeast Asians than to Chinese, Japanese or Koreans. Hence, it is possible that within these two former genetically similar ethnic groups, the ΔTTTCT deletion is not correlated to BMI, or the effect of the deletion is masked by other factors. Future studies with larger samples sizes will be required to determine whether the effect of ΔTTTCT deletion on BMI is restricted to a subset of ethnic groups.

The association between the ΔTTTCT intronic deletion polymorphism in the RGS9 gene and increased BMI was studied in the East Asian population because the deletion was rare in other genetically similar ethnic groups, the ΔTTTCT deletion polymorphism and the identity to a binding motif for the polypyrimidine tract binding protein (PTB) suggested that this naturally occurring allele could affect the splicing of RGS9 mRNA. Indeed, our data shows that the ΔTTTCT deletion polymorphism lies within intron 13. The exons that flank intron 13 code Exons 13 and 14 which encode the RGS domain [17]. The RGS domain is conserved among all the RGS family members and mediates the canonical Gz GAP function [3]. Thus, altered splicing due to the ΔTTTCT deletion polymorphism, such as exon skipping or intron retention, will result in a protein product that is truncated at this region and therefore lacks the important class-defining GAP function. These
results, in turn, suggest that individuals carrying the deletion polymorphism have a higher BMI due to a reduction in the functional levels of brain RGS9-2.

Support for the suggestion that RGS9-2 is important in regulating body-weight is provided by the finding that mice with the RGS9 gene deletion have elevated body weight (Fig. 2) and that conversely overexpressing RGS9-2 in the rat nucleus accumbens (NAc), via herpes simplex virus (HSV)-mediated gene transduction, lowers body weight relative to control animals (Fig. 4). RGS9-2 is expressed in the vast majority of striatal medium spiny neurons [7], which comprise 90–95% of the neurons in the striatum [28]. Hence, the percentage of infected non-target cells that do not normally express RGS9-2 is small and consequently, this technique has been successfully utilized to define physiological and pathophysiological functions for RGS9-2 using both rodent and primate models [7,24].

Indeed, the results presented above mirror those from previous studies, where HSV-mediated RGS9-2 over-expression in the rat striatum has been shown to produce functional responses that are opposite to those exhibited by the RGS9 knockout mice. For example, RGS9 knockout mice exhibit increased cocaine-induced locomotion, while HSV-mediated overexpression of RGS9-2 in rats dampens cocaine-induced locomotion [7]. In addition, RGS9 knockout mice show accelerated development of drug-induced dyskinesia [6], while HSV-mediated overexpression of RGS9-2 in the striatum of rats and monkeys diminished intensity of drug-induced dyskinesia [24].

The specificity of RGS9-2 overexpression effects are highlighted by the parallel experiments with RGS7 and RGS11 [18], closely related members of the R7 RGS protein family. Though RGS11 is thought to be specifically expressed in retinal bipolar neuron, the two proteins have been shown in vitro to act as GTPase accelerating proteins for the same G proteins [18,29,30]. The opposite effects of RGS9-2 and RGS7 and RGS11 overexpression on body weight (Fig. 4) suggest that the effects cannot be attributed solely to the GAP function which is common to the three proteins. While we do not yet understand the mechanism for the opposing action of these proteins on body weight, it is interesting to note that R7 RGS proteins, such as RGS7, RGS9 and RGS11, can compete for their obligate binding partners (Gβ5 and R9AP or R7BP). In fact, knock-out of these binding partners leads to marked reduction in all R7 RGS proteins [31,32,33]. Therefore, one hypothesis explaining the relative increase in weight produced by RGS7 or RGS11 overexpression, is that RGS7 and RGS11 competes with RGS9-2 for the available Gβ5 and R7BP, destabilizing native RGS9-2 protein and producing an effect on weight similar to that seen in RGS9 knockout mice. Results similar to those we report have been observed at the cellular level: previous studies have reported that, despite their structural similarity, RGS7 and RGS11 produce opposite effects to those produced by RGS9 on cellular signaling pathways [34,35].

While there is one report of RGS9 transcript expression in blood lymphocytes [36], all other studies examining the tissue distribution and function of RGS9 gene products have reported only on the expression of RGS9 in the brain and the retina [5,17,37,38]. Thus, the absence of reports of RGS9 expression in peripheral tissue, interpreted in conjunction with, i) our observations with rats demonstrating that RGS9-2 overexpression in the NAc lowers body weight compared to control animals (Fig. 4) and ii) undetectable levels of RGS9 protein expression in fat tissue (Fig. 3B), suggest that the RGS9 acts to regulate body-weight and adiposity via expression in the brain.

RGS9-2 exhibits extremely dense expression in rodent striatum, and pleasure, desire and reward circuits operating in the striatum are thought to be important in obesity and eating disorders [39]. However, the actions of RGS9-2 in regulating body-weight and adiposity are likely to involve alternative striatal connections, since we report that the food intake of the heavier RGS9 knockout mice was similar to that of wild-type mice.

The dopamine-sensitive NAc reward centers are densely inter-connected with the hypothalamus, a brain region that is critical for regulating energy expenditure. Hypothalamic neurons, including orexin [40,41] and melanin concentrating hormone neurons [42], make connections with the NAc, and the NAc can influence hypothalamic functions [43]. Thus, RGS9-2 via expression in the NAc could modulate the activity of hypothalamic centers that control energy expenditure and future experiments will examine the total and resting oxygen consumption in the RGS9 knockout mice. Interestingly, in addition to extremely dense expression in striatum, RGS9-2 is also localized to medial hypothalamus [38]. However, it is clear from our experiments that modulating RGS9-2 levels specifically in the NAc can alter body weight (Fig. 4).

Human genetic studies have linked variations in two other R7 RGS proteins, RGS6 [44] and RGS7 [44] to obesity. The above human genetic studies provide a precedent for R7 RGS family proteins regulating body-weight through their expression in the brain since these proteins have been reliably detected only in the brain or in excitable tissue such as retina and heart [37,45,46].

More recently, it has been reported that mice with a targeted deletion of one copy of the G protein Gβ5 subunit gene are heavier and have increased adiposity when compared to their wild-type counterparts, even though their food intake was not different and their locomotor activity levels were enhanced [47]. Similarly, we found that the food intake of the heavier RGS9 knockout mice was not different from their wild-type littermates, and also observed a small but significant increase in initial activity of the male RGS9 knockout mice when placed in the movement measurement cages (Fig. 2D). Gβ5 protects R7 RGS family proteins from proteolysis and expression of R7 RGS family proteins, including RGS9, is eliminated or hugely reduced in the absence of Gβ5 [18,32]. Therefore our results raise the possibility that the increased adiposity and weight phenotype of heterozygous Gβ5 knockout mice is produced as a result of a Gβ5 knockdown-mediated reduction in brain RGS9-2 protein levels.

Human and mouse studies have also implicated RGS2, 4 and 5, in the regulation of body weight and obesity [48,49,50,51]. However these RGS proteins and RGS9 likely regulate body weight through different mechanisms: RGS2, 4 and 5 are members of a different R4 subfamily of RGS proteins, have a different molecular architecture and strikingly different cellular and tissue expression patterns compared to RGS9 [18,37,52]. It has been reported that knock-in mice homozygous for a mutant Gα2 G protein subunit that does not bind RGS protein are resistant to diet-induced obesity [53]. However, the molecular mechanism underlying the body-weight phenotype of these mice is also likely to be different from that in the RGS9 knockout mice: Gα2, is expressed ubiquitously in peripheral tissues and in many brain regions and does not have the restricted expression pattern of the RGS9 gene products [53].

Previously, altered RGS9-2 levels have been shown to be involved in drug-addiction and the reward response to psycho-stimulants [15] and our results are significant because we implicate the same molecule in the control of body weight and adiposity.

Materials and Methods

Human genotyping

The human genotyping study was approved by the Human Research Protection Program Committee on Human Research of
the University of California, San Francisco. The study application was titled, “Discovery of Gene Mutations and Polymorphisms that Contribute to the Risk of Cardiovascular and Metabolic Disease and Macular Degeneration”. The Institutional Review Board number was 10-00207 and the permit number was 016323. Written informed consent was obtained from all subjects.

DNA samples were analyzed for the presence of the ΔTTTCT deletion polymorphism from participants of East Asian ancestry, in the University of California, San Francisco, Genomic Resource in Arteriosclerosis (GRA) collection, which has been previously described [54,55] and associated height, weight, sex and age data. The body mass index for each subject was calculated using the formula: mass (kg) + (height (m))^2 [25].

The primer sequences used for detecting the ΔTTTCT deletion polymorphism were, forward, 5’-GGCAATAGCTTTTCTGG- and reverse, 5’-TGCCGATGACAGTGACCATGATG (chr17: 63,197,962–63,198,341). Forward primers were fluorescently labeled with 6-FAM (Life Technologies-Invitrogen-Applied Biosystems, Carlsbad, CA). PCR was carried out using Platinum Taq polymerase (Invitrogen). The PCR reaction was performed on 10 to 25 ng of genomic DNA with 2.0 mM MgCl2, 0.25 units Taq, 200 μM dNTPs, 0.02 μM of each primer and PCR buffer supplied with the Taq enzyme. An ABI 3730 (Applied Biosystems) DNA analyzer was utilized for the initial Sanger sequencing visualization and fragment separation. The genotyping results were determined by visualization using STRand [56] while Sequencher (Gene Codes Corporation, Ann Arbor, MI) was used to analyze the sequencing results.

The statistical analysis of the human data was performed using IBM SPSS Statistics (IBM, Armonk, NY) and Systat 12 (Systat Software, Chicago, IL) software.

Splicing reporter minigene assay

The reporter system used to screen for the possible effect on splicing of the ΔTTTCT deletion polymorphism was derived from a plasmid vector containing a 3 exon minigene under the control of the human cytomegalovirus promoter and has previously been described by Wang and colleagues [57]. The first and last exons of the original 3 exon minigene consist of sequences that encode for portions of the enhanced green fluorescent protein (eGFP). These two exons are separated by a small constitutively spliced exon, of the Chinese hamster dihydrofolate reductase (DHFR) gene, together with its flanking introns. The DHFR exon also contains two restriction cloning sites, Xhol and Apai, into which we inserted a synthesized (Epoch Life Sciences, Missouri City, TX) fragment of the human RGS9 gene [17]. A schematic of the minigene construct is shown in Fig. 1A. The inserted RGS9 gene fragment consisted of the final 57 base pairs of exon 13 (Fig. 1A, grey box), followed by a fusion of the first 100 and the last 243 base pairs in the adjacent intron 13 (Fig. 1A, dashed grey bar). The insertion of the regions from intron 13 of the RGS9 gene effectively splits the central DHFR exon of the original minigene into 2 separate exons thus creating a new minigene construct with 4 exons (Fig. 1A). To investigate the putative effect of the ΔTTTCT deletion on splicing, a second construct, identical to the one described above, was made except that the inserted RGS9 gene fragment was synthesized with ΔTTTCT deletion (represented as A in Fig. 1A) at the appropriate intron 13 site. To increase the likelihood of detecting splicing effects of a polymorphism located outside the canonical splicing sites, we weakened the canonical splicing machinery near the 3’ splicing site (C→A, at position -3) and near the 5’ splicing site (G→T, last position in the exon). A similar strategy of weakening splicing signals or adding negative elements to impair the recognition of a test exon has been commonly used to test the effects of silencer or enhancer elements in other studies [21].

Human embryonic kidney cells (HEK293, American Type Culture Collection, Manassas, VA) were separately transfected with equal amounts of the ΔTTTCT deletion positive (+ve) or the ΔTTTCT deletion negative (-ve) minigene constructs described above. The transient transfections were performed using LTX transfection reagent (according to manufacturer’s instructions Invitrogen, Carlsbad, CA).

After 48 hr the cells were harvested and total cellular RNA was isolated using TRIzol reagent (according to manufacturer’s instructions, Invitrogen). Next, the RNA was reverse transcribed (reverse transcriptase enzyme, Sigma Aldrich) at 37 °C for 50 min in a reaction that was primed with random hexamer nucleotides and contained dNTP mixture and RNase inhibitor (Ambion) and buffer provided by the enzyme supplier.

The resulting cDNA was amplified in a PCR reactions (25 cycles) using Taq DNA polymerase (Promega, Madison, WI) and forward (5’ AAATGGGAATCTCCTGGG 3′) and reverse (5’ AAACCTGAACTCTCATT 3′) primers flanking the first and last exon of the minigene such that splicing alterations resulting from exon skipping or intron retention would alter the size of the PCR fragment amplified. The identity of the alternatively spliced gene fragments were confirmed by sequencing.

Rodent studies

Rodent studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental protocols using rats and mice were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center at Dallas or the University of Rhode Island with protocol numbers, 0930-04-02-1 and 242945-1, respectively.

Mouse Weight Measurements

Genotyping of the rgs9−/− mice was described previously [58]. Mice were backcrossed to C57BL/6J mice (Stock Number: 000664, Jackson Laboratory, Bar Harbor, ME) for four generations. An rgs9+/− X rgs9+/− breeding strategy was used to generate wild-type (rgs9+/+), heterozygous (rgs9+/−) and RGS9 knockout (rgs9−/−) littermates. At weaning, mice were tail-clipped for genotyping, ear-tagged, re-caged 4 per cage, in polysulfone cages that were not environmentally enriched (part #: RC71D-UD Udel® Polysulfone, Alternative Design, Siloam Springs, AR, 18.4 cm wide, 29.2 cm deep, 12.7 cm high, 419.35 cm² floor area), with same sex, and switched from an ad libitum 6% fat, breeding diet (catalog #: 7002, Teklad 6% Fat Mouse/Rat Diet, Harlan, Indianapolis, IN) to an ad libitum 4% fat standard laboratory chow diet (catalog #: 7002, Teklad 4% Fat Mouse/Rat Diet, Harlan) and weighed daily until post-weaning day 50.

Mouse Locomotor Activity Measurements

Locomotor activity was measured using an automated system (Med Associates, St. Albans, VT). Mice were placed singly in plastic activity chambers (12×18×33 cm) with 10 pairs of photocell beams dividing the chamber into 10 rectangular fields for measurement of total beam breaks, as described previously [7]. 8 week old mice were placed into the cages and their ambulatory locomotor activity was monitored for 2 hrs. Data are presented as number of beam breaks after 30 min and after 2 hr.
Comparison and Histochemical Analysis of Visceral Adipose Tissue

Epididymal, mesenteric and peri-renal adipose tissue depots were excised from age-matched 2-3.5 month old male g9/+ and g9/- mice, fixed in 10% (vol/vol) buffered formalin and then separately weighed. The mass of peri-renal adipose tissue obtained from each mouse was too small to be accurately weighed and therefore the peri-renal depot was not used in the subsequent analyses.

For histological studies of the adipose tissues, the formalin fixed tissues were dehydrated through graded ethanol, and embedded in paraffin. Sections were cut at 4 μm and stained with hematoxylin and eosin according to standard staining protocols [59].

Bright field images of hemotoxylin and eosin stained sections of mouse fat pads were captured using a 40X objective and Nikon (Melville, NY) Eclipse TS100 microscope. The adipocyte images were analyzed using the automated object/cell counting and analysis features of NIS-Elements software (Nikon). The software parameters were manually adjusted to maximize the detection and outlining of adipocytes borders in each image. Misidentified cells and partial cell images at the edges at the edges of each picture were manually excluded prior to analysis.

Western blotting

Epididymal fat pads and striatal tissue was dissected from wild-type (RGS9+/+) mice and Dounce homogenized in 1X phosphate buffered saline (PBS) containing a cocktail of protease inhibitors (catalog #: S8330, Sigma-Aldrich, St. Louis, MO), solubilized in PBS containing 2% w/v sodium dodecyl sulfate (SDS) and sonicated to reduce sample viscosity. Protein concentration of resulting samples was determined using a BCA protein assay kit (catalog #: # I116707, Pierce/Thermo Scientific, Rockford, IL).

60 μg of total protein, diluted in SDS-sample buffer containing 2% SDS, 100 mM Tris-HCl, and 50 mM diethiothreitol (DTT) was loaded per well of a 10-well mini-gel plate (mini-PROTEAN 1-dimensional electrophoresis system, Bio-Rad, Hercules, CA), resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene fluoride (PVDF) membrane and probed using a previously validated primary polyclonal sheep antibody that specifically recognizes both long and short RGS9 isoforms [23] and an anti-sheep horse-radish peroxidase (HRP)-conjugated secondary (Jackson ImmunoResearch, West Grove, PA). A luminescent signal from the HRP-conjugated secondary was elicited using a chemiluminescent HRP substrate (catalog #: S8830, Sigma-Aldrich, St. Louis, MO), solubilized in PBS containing 2% SDS, 100 mM Tris-HCl, and 50 mM dithiothreitol (DTT) was loaded per well of a 10-well mini-gel plate (mini-PROTEAN 1-dimensional electrophoresis system, Bio-Rad, Hercules, CA), resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene fluoride (PVDF) membrane and probed using a previously validated primary polyclonal sheep antibody that specifically recognizes both long and short RGS9 isoforms [23] and an anti-sheep horse-radish peroxidase (HRP)-conjugated secondary (Jackson ImmunoResearch, West Grove, PA). A luminescent signal from the HRP-conjugated secondary was elicited using a chemiluminescent HRP substrate (catalog #: S8830, Sigma-Aldrich, St. Louis, MO), solubilized in

Table S1 Table describing the ethnic, sex and ΔΔTCT deletion positive (Δ+) and ΔΔTCT (Δ-) deletion negative composition of the human study sample.

Table S2 Detailed table describing the East Asian study population.

Acknowledgments

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NCRR, NIMH or the NIH.

Author Contributions

Conceived and designed the experiments: JLW SJG WGF SPJ JC VZ AK. Performed the experiments: JLW MS JC DT VC CT. Analyzed the data: JLW MS JC RLD DT SPJ VZ. Contributed reagents/materials/ analysis tools: SCR RLN JPK MJM CRP. Wrote the paper: JLW SJG AK.

Supporting Information

Table S2 Detailed table describing the East Asian study population.

Table S2 Detailed table describing the East Asian study population.
References

1. Bodnar RJ (2004) Endogenous opioids and feeding behavior: a 30-year historical perspective. Peptides 25: 697–725.

2. Gold SJ, Roussel M, Wang J (2007) Mesulam D1 receptor knockout mouse. J Biol Chem 282: 12744–12748.

3. Willars GB (2006) Mammalian RGS proteins: multifunctional regulators of cellular signalling. Semin Cell Dev Biol 17: 363–376.

4. Sethakorn N, Yau DM, Dulun NO (2010) Non-canonical functions of RGS proteins, Cell Signal 22: 1274–1281.

5. Rahman Z, Gold SJ, Potenza MN, Cowan CW, Ni YG, et al. (1999) Cloning and characterization of RGS9-2: a striatal-enriched alternatively spliced product of the RGS8 gene. J Neurosci 19: 2016–2026.

6. Kovoor A, Seyffarth P, Ebert J, Barghshoon S, Chen CK, et al. (2005) D2 dopamine receptors colocalize regulator of G-protein signaling 9-2 (RGS9-2) via the RGS9 DEP domain, and RGS9 knockout mice develop dyskinesias associated with dopamine pathways. J Neurosci 25: 2157–2162.

7. Rahman Z, Schwartz J, Gold SJ, Zachariou V, Wein MN, et al. (2005) RGS9 modulates dopamine signaling in the basalganglia. Neuron 38: 941–952.

8. Celver J, Sharma M, Kovoor A (2010) RGS9-2 mediates specific inhibition of agonist-induced internalization of D2-dopamine receptors. J Neurochem 114: 739–749.

9. Cabrera-Vera TM, Hernandez S, Earls LR, Medkova M, Sundgren-Petri MM (2011) Ancient voyaging and neomigration of CD4+ naïve and regulatory T cells. Immunol. 115: 179–188.

10. Posokhova E, Wydeven N, Allen KL, Wickman K, Martemyanov KA (2010) RGS6/Gsa5 complex regulates AKCh gating kinetics in striatal neurons and modulates parasympathetic regulation of heart rate. Circ Res 107: 1340–1349.

11. Yang J, Huang J, Maity B, Gao Z, Lorca RA, et al. (2010) RGS6, a modulator of G protein signaling-insensitive G184S Gnai2 allele. Diabetes 59: 77–85.

12. Bubser M, Fadel JR, Jackson LL, Meador-Woodruff JH, Jing D, et al. (2005) Dopaminergic regulation of locomotor activity. Eur J Neurosci 21: 2959–3001.

13. Baldo BA, Daniel KA, Berridge CW, Kelley AE (2004) Overlapping distributions of orexin/hypocretin- and dopamine-beta-hydroxylase-immunoreactive fibers in rat brain regions regulating arousal, motivation, and stress. J Comp Neurol 440: 230–251.

14. Saito Y, Cheng M, Leslie FM, Civelli O (2001) Expression of the melanin-concentrating hormone (MCH) receptor mRNA in the rat brain. J Comp Neurol 455: 26–40.

15. Kelley AE, Baldo BA, Pratt WE, Will MJ (2005) Corticotropin-releasing-hormone and food motivation: integration of energy, action and reward. Physiol Behav 86: 773–795.

16. Aissani B, Perrus E, Lapointe G, Chagoun YC, Bouchard P, et al. (2006) A quantitative trait locus for body fat on chromosome 1q33 in French Canadians: linkage and association studies. Obesity Silver Spring 14: 1603–1615.

17. Posokhova E, Wydeven N, Allen KL, Wickman K, Martemyanov KA (2010) RGS6/Gsa5 complex regulates AKCh gating kinetics in striatal neurons and modulates parasympathetic regulation of heart rate. Circ Res 107: 1340–1349.

18. Wang Q, Levay K, Chang M, Huang J, Ho CY, Richard JM, et al. (2011) Targeted deletion of one or two copies of the G protein [beta] subunit G[beta]5 gene has distinct effects on body weight and behavior in mice. FASEB J. 25: 3906–3917.

19. Soares P, Rito T, Trejaut J, Soffiller P, Martemyanov KA (2007) Localization and differential interaction of R7 GRS proteins with their membrane anchors R7BP and R9AP in neurons of vertebrate retina. Mol Cell Neurosci 35: 311–319.

20. Yang J, Huang J, Maity B, Gao Z, Lorca RA, et al. (2010) RGS6, a modulator of G protein signaling-insensitive G184S Gnai2 allele. Diabetes 59: 77–85.

21. Shiffman D, Ellis SG, Rowland CM, Malloy MJ, Luke MM, et al. (2005) Effect of RGS9-2 gene polymorphism on diet-induced obesity and improved insulin sensitivity in mice with a regulator of G-protein signaling-insensitive G184S Gnai2 allele. Diabetes 54: 4371–4378.

22. Baldo BA, Daniel KA, Berridge CW, Kelley AE (2004) Overlapping distributions of orexin/hypocretin- and dopamine-beta-hydroxylase-immunoreactive fibers in rat brain regions regulating arousal, motivation, and stress. J Comp Neurol 440: 230–251.

23. Nunn C, Zhao P, Zou MX, Summers K, Guglielmo CG, et al. (2011) Resistance to diet-induced obesity and improved insulin sensitivity in mice with a regulator of G-protein signaling-insensitive G184S Gna2 allele. Diabetes 57: 77–85.

24. Shiffman D, Ellis SG, Rowland CM, Malloy MJ, Luke MM et al. (2005) Identification of four gene variants associated with myocardial infarction. Am J Hum Genet 76: 596–605.

25. Pullinger CR, Aoizrabiait BE, Mouwbyen Y, Iurachal V, Sibajrns E, et al. (2008) An adipoprotein A-2 gene SNP is associated with marked hypertrophy-phenotype differences among Asian-American patients. J Lipid Res 49: 1844–1854.

26. Toonen RJ, Hughes S (2001) Increased throughput for fragment analysis in an ABI PRISM 377 automated sequencer using a membrane column and STRand software. Biotechniques 31: 1320–1324.
57. Wang Z, Rolish ME, Yeo G, Tung V, Mawson M, et al. (2004) Systematic identification and analysis of exonic splicing silencers. Cell 119: 831–845.
58. Chen CK, Burns ME, He W, Wensel TG, Baylor DA, et al. (2000) Slowed recovery of rod photoreceptor in mice lacking the GTPase accelerating protein RGS9-1. Nature 403: 557–560.
59. Kiernan J (2008) Histological and Histochemical Methods: Theory and Practice. Cold Spring HarborNY: Cold Spring Harbor Laboratory Press. 606 p.
60. Carlezon WA, Jr., Boundy VA, Haile CN, Lane SB, Kalb RG, et al. (1997) Sensitization to morphine induced by viral-mediated gene transfer. Science 277: 812–814.
61. Carlezon WA, Jr., Nestler EJ, Neve RL (2000) Herpes simplex virus-mediated gene transfer as a tool for neuropsychiatric research. Crit Rev Neurobiol 14: 47–67.
62. Carlezon WA, Jr., Neve RL (1999) Expression of multiple proteins within single primary cortical neurons using a replication deficient HSV vector. Biotechniques 27: 1156–1160.
63. Kalivas PW, Duffy P (1990) Effect of acute and daily cocaine treatment on extracellular dopamine in the nucleus accumbens. Synapse 5: 1156–1166.
64. Rouge-Pont F, Usiello A, Benoit-Marand M, Gonon F, Piazza PV, et al. (2002) Changes in extracellular dopamine induced by morphine and cocaine: crucial control by D2 receptors. J Neurosci 22: 3293–3301.
65. Uhl GR, Hall FS, Sora I (2002) Cocaine, reward, movement and monoamine transporters. Mol Psychiatry 7: 21–26.
66. Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. San DiegoCA: Academic Press.
67. Neve RL, Neve KA, Nestler EJ, Carlezon WA, Jr. (2005) Use of herpes virus amplicon vectors to study brain disorders. Biotechniques 39: 381–391.