



Genetic deletion of regulator of G-protein signaling 4 (RGS4) rescues a subset of fragile X related phenotypes in the FMR1 knockout mouse

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ABSTRACT

Fragile X syndrome (FXS), the most common cause of inherited mental retardation, is caused by the loss of the mRNA binding protein, FMRP. Persons with FXS also display epileptic seizures, social anxiety, hyperactivity, and autistic behaviors. The metabotropic glutamate receptor theory of FXS postulates that in the absence of FMRP, enhanced signaling through G-protein coupled group I metabotropic glutamate receptors in the brain contributes to many of the abnormalities observed in the disorder. However, recent evidence suggests that alterations in cellular signaling through additional G-protein coupled receptors may also be involved in the pathogenesis of FXS, thus providing impetus for examining downstream molecules. One group of signaling molecules situated downstream of the receptors is the regulator of G-protein signaling (RGS) proteins. Notably, RGS4 is highly expressed in brain and has been shown to negatively regulate signaling through Group I mGluRs and GABA_B receptors. To examine the potential role for RGS4 in the pathogenesis of FXS, we generated FXS/RGS4 double knockout mice. Characterization of these mice revealed that a subset of FXS related phenotypes, including increased body weight, altered synaptic protein expression, and abnormal social behaviors, were rescued in the double knockout mice. Other phenotypes, such as hyperactivity and macroorchidism, were not affected by the loss of RGS4. These findings suggest that tissue and cell-type specific differences in GPCR signaling and RGS function may contribute to the spectrum of phenotypic differences observed in FXS.

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Introduction

Fragile X syndrome (FXS) results from a trinucleotide repeat expansion in the 3' untranslated region of the X-linked FMR1 gene. This expanded repeat induces hypermethylation of the promoter and subsequent loss of the encoded protein, the mRNA binding protein fragile X mental retardation protein (FMRP). Individuals with FXS exhibit a spectrum of abnormalities including mild to moderate mental retardation, anxiety, hyperactivity, autistic behaviors and seizures (Hagerman et al., 2009; Hall et al., 2009; Walter et al., 2009). FMRP normally functions to regulate the translation of a large number

of mRNAs, most of which are unknown, and many of which are regulated at or near synapses (Feng et al., 1997; Weiler and Greenough, 1999; Napoli et al., 2008; Edbauer et al., 2010). The metabotropic glutamate receptor (mGluR) theory of FXS postulates that protein-synthesis dependent downstream signaling of group I mGluRs is enhanced in the absence of FMRP (Bear et al., 2004). While enhanced group I mGluR signaling may account for some FXS phenotypes (Bear et al., 2004), recent evidence implicates additional G-protein coupled receptors (GPCRs), including acetylcholine (Volk et al., 2007), dopamine (Wang et al., 2008), and GABA_B receptors (Zupan and Toth, 2008; Pacey et al., 2009) in the pathogenesis of FXS.

Regulator of G-protein signaling (RGS) proteins are a large family of more than 36 mammalian proteins that play an important role in fine tuning GPCR signaling in different tissues. RGS proteins attenuate GPCR signaling by acting as GTPase activating proteins (GAPs) of the G α subunits of heterotrimeric G-proteins; RGS proteins accelerate the GTP to GDP cycle thereby reducing GPCR-mediated signaling. Members of the R4/B subclass of RGS proteins are typified by a ~210 amino acid GAP domain capable of inhibiting Gi and/or Gq

Abbreviations: dKO, double knockout; FMRP, fragile X mental retardation protein; FXS, fragile X syndrome; GAP, GTPase activating protein; GIRK, G-protein coupled inwardly rectifying K⁺ channel; GPCR, G-protein coupled receptor; KO, knockout; mGluR, metabotropic glutamate receptor; PND, postnatal day; RGS, regulator of G-protein signaling; WT, wild-type.

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function, that is flanked by short amino- and carboxyl-termini (for review see Bansal et al., 2007). R4 subgroup proteins can thus inhibit GPCR-mediated activation of a variety of downstream effector pathways including phospholipase C, mitogen-activated protein kinases, and G-protein coupled inwardly rectified K⁺ channel (GIRK) channels. Moreover, some RGS proteins can directly modulate intracellular signaling pathways via their non-GAP domains through interaction with other signaling effectors (Shi et al., 2001; Sinnarajah et al., 2001; also see Nguyen et al., 2009).

Altered function of the prototypical R4/B group member, RGS4, has been implicated in the pathogenesis of schizophrenia and heart disease (Riddle et al., 2005; Chowdari et al., 2008), two diseases associated with altered GPCR activity. RGS4 can dampen signaling of GPCRs coupled to G α q-mediated stimulation of IP₃ production and intracellular calcium release (Hepler et al., 1997; Yan et al., 1997) as well as Gi-mediated regulation of GIRK channel activity (Cifelli et al., 2008; Doupnik, 2008). To investigate a potential role for RGS4 in the regulation of GPCR signaling in FXS, we examined FMR1/RGS4 double knockout mice and compared them with FXS knockout mice (called FMR1 mice), RGS4 knockouts, and wild-type (WT) mice. Given that mGluR signaling is enhanced in FXS, and RGS4 has been shown to attenuate group I mGluR signaling (Saugstad et al., 1998), we hypothesized that FMR1/RGS4 double knockouts would exhibit exacerbated symptoms of FXS. Instead, we found that RGS4 knockout reduces susceptibility of FMR1 knockout mice to audiogenic seizures and that this effect is mediated, at least in part, by increased signaling through GABA_B receptors (Pacey et al., 2009). The objective of the current study was to examine the effect of RGS4-deficiency on a range of other phenotypes identified in the FMR1 knockout mouse model of FXS. We found that loss of RGS4 rescues a number of the fragile X related phenotypes in the FMR1 knockout mouse, implicating decreased GPCR signaling as a contributor to FXS pathogenesis.

Results

RGS4 mRNA is not changed in fragile X knockout mice

RGS4 mRNA expression was examined in the hippocampus and cerebral cortex of postnatal day (PND) 12, PND 30 and adult (2–4 months) WT and FMR1 knockout mice by quantitative RT-PCR. Compared to WT mice, FMR1 knockout mice showed no statistically significant difference in RGS4 mRNA levels in the cortex or hippocampus at any developmental time point (Fig. 1). Although not statistically different, it is interesting to note that RGS4 mRNA levels were elevated in both brain regions at all time points examined. These

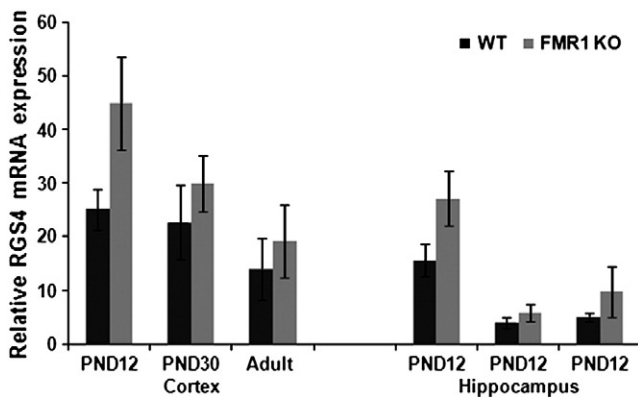


Fig. 1. RGS4 mRNA expression in FMR1 knockout mice. RGS4 mRNA levels were examined in the hippocampus and cerebral cortex of PND 12, PND 30 and adult FMR1 knockout mice by quantitative RT-PCR. Compared to wild-type mice, RGS4 mRNA was not statistically different in FMR1 knockout mice in either brain region at any time point examined.

findings differ from those of a previous report where *in situ* hybridization data indicated that RGS4 mRNA levels were decreased in hippocampal pyramidal neurons and in the retrosplenial cortex but not in the piriform cortex of 10 day old but not in adult FMR1 mice (Tervonen et al., 2005). The reason for this discrepancy may relate to subtle differences in the age of the animals used in the two studies, or to the lower degree of spatial resolution in real-time RT-PCR compared to *in situ* hybridization.

Body weight is normalized in FMR1/RGS4 knockout mice

Persons with FXS exhibit accelerated pre-adolescent growth (Loesch et al., 1988) and a previous study demonstrated a similar increase in total body weight in FMR1 mice (Dolen et al., 2007). At PND 30, total body weight of RGS4 knockout males was not different from WT mice ($p > 0.05$; Fig. 2A). Male FMR1 knockout mice showed 17% and 13% increases in total body weight over WT and RGS4 knockout mice respectively ($p < 0.001$ and $p < 0.05$). Notably, the body weight of FMR1/RGS4 double knockout mice was significantly lower than the FMR1 mice ($p < 0.05$), but not significantly different from wild-type or RGS4 knockouts ($p > 0.05$). No effect of genotype on body weight was observed in female mice at PND 30 (Fig. 2B). These results demonstrate that knockout of RGS4 normalizes the increased body weight phenotype in male FMR1 knockout mice.

Testicular weight is unaffected by RGS4 knockout

Post-pubescent males with fragile X demonstrate macroorchidism, a characteristic which is also present in FMR1 knockout mice (Yan et al., 2004). Testicular weight was analyzed in adult (2–4 months old) male wild-type ($n = 9$), RGS4 knockout ($n = 5$), FMR1 knockout ($n = 5$) and FMR1/RGS4 double knockout mice ($n = 8$). The combined weight of both testes was measured and expressed as a percentage of total body weight. FMR1 knockout mice showed a 41% increase in testicular weight compared to WT animals (Fig. 2C; $p < 0.0001$). Testis weight was increased by 35% in double knockouts compared to WT ($p < 0.0001$) and double knockouts did not differ from FMR1 knockouts ($p > 0.05$). RGS4 knockout mice did not differ from WT ($p > 0.05$). This result indicates that eliminating RGS4 does not affect testicular weight in FMR1 knockout mice.

FMR1/RGS4 double knockouts show normal PSD-95 expression

PSD-95 is a post-synaptic density scaffold protein whose mRNA has been shown to be a target of FMRP (Muddashetty et al., 2007; Zalfa et al., 2007). In the absence of FMRP, PSD-95 mRNA is unstable and PSD-95 mRNA and protein levels are decreased in the brains of FMR1 knockout mice (Zalfa et al., 2007). We measured PSD-95 protein levels in forebrain homogenates from PND 12 WT ($n = 11$), RGS4 knockout ($n = 7$), FMR1 ($n = 7$), and FMR1/RGS4 double knockout mice ($n = 8$) by quantitative Western blotting (Fig. 3). In FMR1 mice, PSD-95 expression was significantly decreased to $43 \pm 7\%$ of wild-type levels ($p < 0.001$ compared to 100% wild-type levels). This decrease was reversed in FMR1/RGS4 double knockouts, which showed PSD-95 expression at $100 \pm 4\%$ of wild-type levels ($p > 0.05$ compared to wild-type; $p < 0.001$ compared to FMR1 KO). RGS4 knockout mice showed levels of PSD-95 similar to WT mice ($90 \pm 9\%$, $p > 0.05$). We also found a similar decrease in PSD-95 expression in the forebrains of adult FMR1 knockout mice ($67 \pm 4.4\%$ of WT; $p < 0.01$; Suppl. Fig. 1). This decrease was also rescued in FMR1/RGS4 double knockout mice ($99 \pm 5.0\%$ of WT; $p > 0.05$ compared to WT; $p < 0.01$ compared to FMR1 KO). This result demonstrates that reduction of RGS4 expression restores normal PSD-95 protein expression levels in the forebrain of postnatal day 12 and adult fragile X mice.

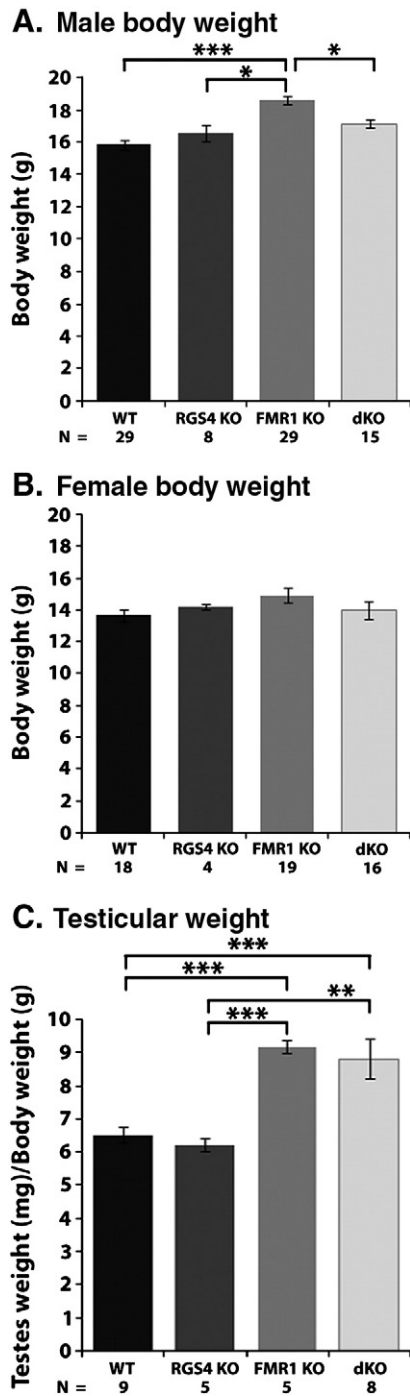


Fig. 2. Body and organ weights. A, At PND 30, male FMR1 knockout mice had higher body weights than wild-type mice. Body weights of RGS4 KO and FMR1/RGS4 double knockout male mice were not different from wild-type, but were significantly lower than FMR1 KO mice. B, In female mice no differences in body weight among the genotypes was observed at PND 30. C, Adult male FMR1 and FMR1/RGS4 double knockout mice had significantly increased testicular weights compared to wild-type mice; testicular weights of RGS4 KO mice did not differ from wild-types. One-way ANOVA followed by Tukey's post-hoc analysis was used to determine statistical significance. N values for each genotype are listed below the corresponding bars. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

GABA_A receptor expression is normalized in FMR1/RGS4 double knockouts

Alterations in the expression of mRNAs coding for GABA enzymes and GABA_A receptors have been reported in FMR1 knockout mice (El Idrissi et al., 2005; D'Hulst et al., 2006; Centonze et al., 2008; D'Hulst

et al., 2009). We have shown that several subunits of the GABA_A receptor proteins are down-regulated in the brains of immature FMR1 knockout mice (Adusei et al., 2010). In the present study, we compared the expression of the $\alpha 1$ subunit of the GABA_A receptor in WT ($n = 10$), RGS4 knockout ($n = 7$), FMR1 knockout ($n = 6$) and double knockout animals ($n = 8$) (Figs. 4A and B). In the forebrains of PND 12 FMR1 mice, expression of the GABA_A receptor $\alpha 1$ subunit was reduced to $41 \pm 8\%$ of wild-type levels ($p < 0.001$). FMR1/RGS4 double knockout mice showed GABA_A $\alpha 1$ levels similar to wild-type ($103 \pm 6\%$; $p > 0.05$) but statistically increased compared to FMR1 mice ($p < 0.001$). GABA_A $\alpha 1$ expression in RGS4 knockout mice was reduced but was not significantly different from wild-type levels ($85 \pm 8\%$, $p > 0.05$). These results demonstrate that genetic deletion of RGS4 can restore GABA_A receptor expression to wild-type levels in FMR1 knockout mice.

GABA_B receptor expression is reduced in FMR1 knockout mice

GABA_B receptors are heteromeric G-protein coupled receptors that require coupling of two distinct subunits, the R1 subunit which contains the ligand binding site, and R2, which mediates transport of the complex to the cell surface and interaction with G proteins to form functional receptors. We analyzed the expression of both subunits of the GABA_B receptor in forebrain homogenates from PND 12 (Figs. 4C and D) and adult (Suppl. Fig. 2) WT, FMR1 knockout, RGS4 knockout and double knockout animals. As we previously reported (Adusei et al., 2010), no significant differences in GABA_BR2 expression were detected in any of the genotypes at either time point (Fig. 4D and Suppl. Fig. 2B). Surprisingly, GABA_BR1 expression was reduced to $50 \pm 6\%$ of WT expression ($p < 0.05$; Fig. 4C) in FMR1 knockout mice. GABA_BR1 expression in FMR1/RGS4 double knockouts was $75 \pm 11\%$ of WT and was not statistically different from either WT or FMR1 knockout mice ($p > 0.05$). Interestingly, GABA_BR1 expression was significantly elevated in RGS4 knockout mice ($163 \pm 12\%$ of WT) compared to all other genotypes ($p < 0.001$). In adult forebrain, FMR1 knockouts showed a slight, but significant, decrease in R1 expression ($78 \pm 4\%$ of wild-type, $p < 0.05$; Suppl. Fig. 2A). There was no difference in GABA_BR1 expression in adult double knockout animals as compared to wild-type ($84 \pm 5\%$ of wild-type, $p > 0.05$). The expression of GABA_BR1 remained significantly elevated ($170 \pm 10\%$, $p < 0.001$) in adult RGS4 knockout forebrain. These results demonstrate (a) reduced expression of the GABA_BR1 subunit in the forebrains of FMR1 mice as early as 12 days postnatal that persists into adulthood, (b) that R1 expression is dramatically elevated in RGS4 knockout animals, and (c) that FMR1/RGS4 double knockouts show levels of GABA_BR1 protein expression similar to WT mice.

RGS4 knockout rescues social behavior impairment in fragile X mice

Tube test for social dominance

Social anxiety is a common characteristic of the human fragile X phenotype (Hessl et al., 2006; Hall et al., 2009) and previous studies have demonstrated abnormal social behaviors in FMR1 knockout mice (Spencer et al., 2005; McNaughton et al., 2008). The tube test can be used to measure social dominance in mice (Lindzey et al., 1961; Shahbazian et al., 2002; Spencer et al., 2005). Two mice of different genotypes are placed in a clear PVC tube and allowed to approach each other simultaneously; the mouse that stays in the tube the longest is considered the "winner" and the more socially dominant mouse. We tested all combinations of WT, FMR1 knockout, RGS4 knockout, and FMR1/RGS4 double knockout males in the tube test and tallied the number of wins by each genotype against each opponent. When paired with WT mice, FMR1 knockout mice won significantly fewer matches than expected by chance (Fig. 5A; 14/57 wins; $p < 0.001$). FMR1/RGS4 double knockouts won 64% of matches (49/77 wins) against FMR1 knockouts (Fig. 5E; $p = 0.017$), but did not differ significantly from wild-types (Fig. 5C; 16/36 wins; $p = 0.505$).

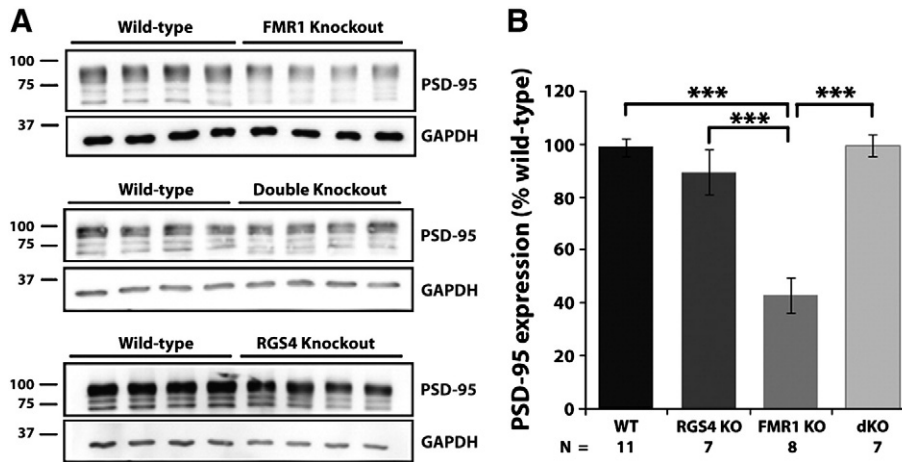


Fig. 3. Changes in the expression of the synaptic scaffold protein PSD-95. Expression of PSD-95 was examined by Western blots of forebrain samples from 12 day old mice. A, Representative Western blots of PSD-95 (top panel; ~95 kDa) expression in wild-type, FMR1 knockout, RGS4 knockout and double knockout mice. GAPDH (bottom panel; 37 kDa) was used as a loading control. B, Summary of PSD-95 expression. PSD-95 was normalized to GAPDH; RGS4 KO, FMR1 KO and double knockout levels are expressed as percent of wild-type expression levels. PSD-95 expression was decreased in FMR1 KO mouse forebrain, compared to wild-type at PND 12. PSD-95 levels in FMR1/RGS4 double KO mice were statistically increased compared to FMR1 knockout and were not different from wild-type. *** $p < 0.001$.

This suggests that knockout of RGS4 can correct the social interaction phenotype of FMR1 mice. Interestingly, RGS4 knockouts won significantly fewer matches against wild-type (Fig. 5B; 8/27 wins; $p = 0.03$) and double knockouts (Fig. 5F; 6/27; $p = 0.004$), but did not

differ from FMR1 knockouts (Fig. 5D; 13/27 wins; $p = 0.847$). Together, these results suggest that, individually, knockout of FMR1 or RGS4 results in abnormal social behavior, but that combining both mutations restores normal social behavior in mice.

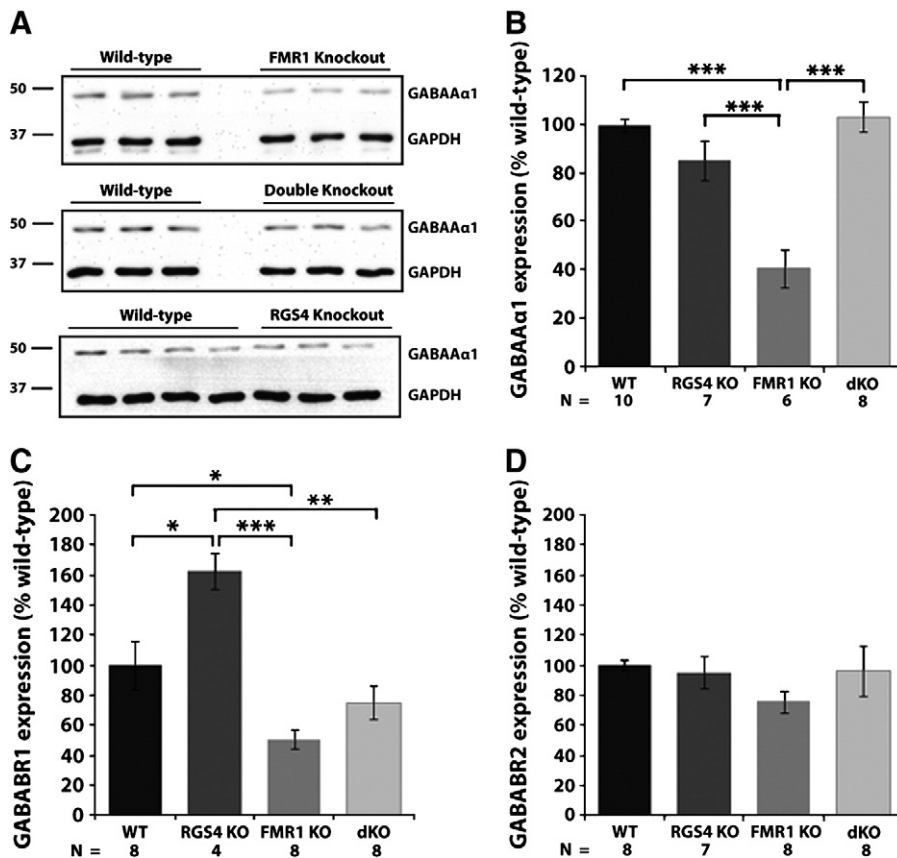


Fig. 4. GABA receptor subunit expression. Expression of the GABA_A receptor α1 subunit and of the GABA_B receptor R1 and R2 subunits were examined by Western blotting in the forebrains of postnatal day 12 mice. A, Representative Western blots of GABA_A receptor α1 (top panel; ~50 kDa) expression in wild-type, FMR1 knockout, RGS4 knockout and double knockout mice. GAPDH (bottom panel; 37 kDa) was used as the loading control. B–D, Summary of the expression of GABA receptor subunits. The blots were normalized to GAPDH expression; RGS4 knockout, FMR1 knockout and double knockout levels are expressed as percent of wild-type expression levels. B, GABA_A receptor α1 subunit expression was decreased in FMR1 KO compared to wild-type mice. In FMR1/RGS4 double knockout animals, GABA_A receptor α1 subunit expression was not different from wild-type but was statistically increased compared to FMR1 knockouts. C, GABA_BR1 expression was significantly decreased in FMR1 KO mice and significantly increased in RGS4 knockouts compared to wild-types. FMR1/RGS4 double knockouts displayed R1 expression that was intermediate between wild-type and FMR1 knockout, but was not significantly different from either genotype. D, GABA_BR2 expression was not different from WT for FMR1 KO, RGS4 KO or double KO mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

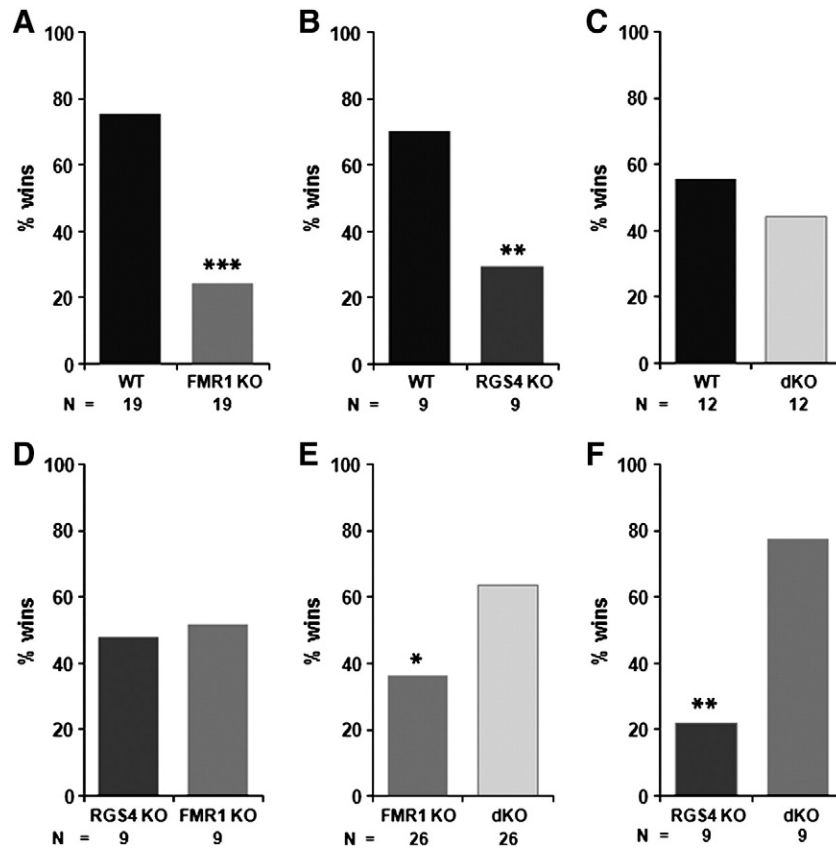


Fig. 5. Tube test for social dominance. Adult male wild-type, FMR1 knockout, RGS4 knockout and FMR1/RGS4 double knockout mice were paired with a mouse of a different genotype and placed head to head inside a clear PVC tube. A, FMR1 knockout mice won significantly fewer matches than expected by chance when paired with wild-type mice (Chi square test). B, RGS4 knockout mice also won fewer matches than wild-types. C, FMR1/RGS4 double knockouts did not differ from wild-type. D, FMR1 knockout and RGS4 knockout mice did not differ in number of matches won. E, Double knockouts won significantly more matches than FMR1 knockouts. F, Double knockouts won significantly more matches than RGS4 knockouts. These results suggest a social dominance hierarchy of WT = dKO > RGS4 KO = FMR1 KO. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Conditioned place preference

The conditioned place preference paradigm is a well known assay used to measure the motivation of an animal. To further characterize social behavior in the four lines of mice we employed a modified conditioned place preference paradigm in which mice were exposed to the scent of an unknown mouse. In this paradigm, animals were conditioned to two distinct environments, one dark environment with a smooth floor and one light environment with a mesh floor separated by a removable partition. On the test day, animals were allowed free access to either environment (the stimulus paired environment or the neutral environment) and a change score was calculated by subtracting the time spent in the stimulus side from the time spent in the neutral side. A positive score denotes a preference for the stimulus, whereas a negative score indicates an aversion.

Wild-type, FMR1, RGS4 knockout and FMR1/RGS4 knockout male mice were tested in this paradigm (Fig. 6). Prior to analysis, we examined whether any group had a light/dark preference and found that none did (wild-type $t(14) = 1.42$, $p = 0.18$; FMR1 knockout, $t(14) = 0.09$, $p = 0.93$; RGS4, $t(14) = 1.69$, $p = 0.11$; and double KO, $t(14) = 1.60$, $p = 0.13$). Wild-type mice showed a change score of 98.38 ± 36.43 ms, indicating a preference for the scent-paired side. In contrast, FMR1 knockout (change score -81.93 ± 33.09 ms) and RGS4 knockout (change score -151.15 ± 46.86 ms) animals showed an aversion to the scent-paired side. Similar to WT mice, FMR1/RGS4 double knockouts also showed a slight preference for the scent-paired side (change score 57.9 ± 55.01 ms). A one way analysis of variance was conducted and the main effect of genotype was statistically significant ($F(3,28) = 7.28$, $p = 0.001$). Pairwise comparisons using a

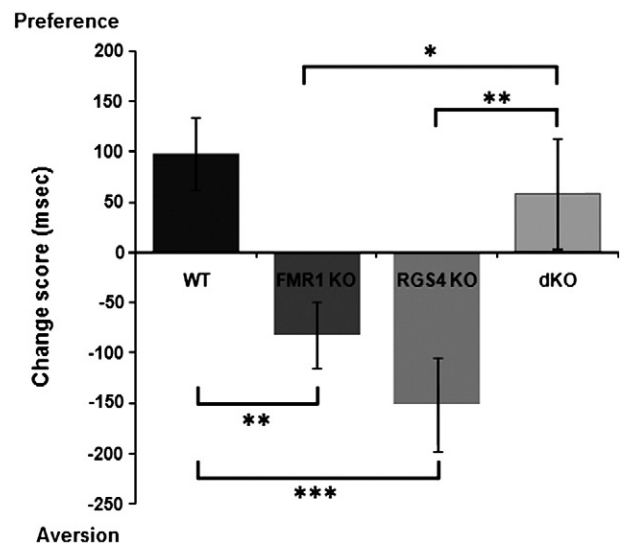


Fig. 6. Conditioned place preference. Adult male mice were conditioned for 4 days to the scent of an unknown mouse. Mice were tested on the fifth day and a change score (time spent on scent-paired side minus time spent on neutral side) was calculated. A positive change score indicates a preference for the scent of the other mouse, while a negative score indicates an aversion. Wild-type animals demonstrated a preference for the scent-paired chamber, while FMR1 knockout and RGS4 knockout mice had an aversion to the scent of another mouse. FMR1/RGS4 double knockout mice spent statistically more time in the scent-paired side indicating a rescue of social avoidance behavior in the double knockouts. $N = 8$ for all four genotypes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

post-hoc Bonferroni analysis demonstrated a statistically significant increase in the change score of WT mice as compared to FMR1 knockouts ($p < 0.01$) and RGS4 knockouts ($p < 0.001$). Interestingly, FMR1/RGS4 double knockout mice did not differ from wild-type ($p > 0.05$) but spent significantly more time in the scent-paired side than FMR1 knockout ($p < 0.05$) and RGS4 knockouts ($p < 0.01$). There was no difference in the change score between FMR1 knockout and RGS4 knockout mice ($p > 0.05$). This result further indicates social avoidance behaviors in both the FMR1 knockout and RGS4 knockout mice, and as seen in the tube test, these behavioral changes are rescued in FMR1/RGS4 double knockout mice.

Open field behavior is not rescued by RGS4 knockout

Hyperactivity is common among persons with FXS and FMR1 knockout mice display increased locomotor activity and exploratory behavior when tested in the open field (Restivo et al., 2005; Yan et al., 2005). We examined the motor activity behavior of six week old (40–45 days) WT ($n = 12$), FMR1 knockout ($n = 12$), RGS4 knockout ($n = 13$) and FMR1/RGS4 double knockout ($n = 12$) mice in the open field over 60 min. Compared to WT mice, FMR1 knockouts showed increased locomotor activity with statistically significant increases in total activity ($p < 0.05$; Figs. 7A and B) and distance traveled ($p < 0.001$; Fig. 7C). Locomotor activity was examined over time by breaking up the testing period into three 20 minute blocks (Fig. 7B). A two-way repeated measures ANOVA revealed a genotype \times time interaction ($p = 0.0157$; $F = 2.786$) and main effects of time ($p < 0.0001$; $F = 54.61$) and genotype ($p < 0.0001$; $F = 10.22$). Post-hoc Bonferroni analysis was conducted to compare each genotype to all others. The

FMR1 knockout and double knockout mice were hyperactive at all time points as compared to wild-type ($p < 0.001$ and $p < 0.01$ respectively) and were not statistically different from each other at any time point ($p > 0.05$). Interestingly, RGS4 knockout mice showed elevated levels of total activity in the first and second segments of testing (Fig. 7B, 0–20 min, $p < 0.01$; 20–40 min, $p < 0.05$) as compared to WT mice. However, in the final 20 min of testing, total activity of RGS4 knockout mice was not different from WT ($p > 0.05$) but significantly lower than FMR1 knockout ($p < 0.01$) and double knockout mice ($p < 0.01$).

Exploratory behavior, as measured by total rearing, was also increased in FMR1 knockout mice ($p < 0.05$; Fig. 7D). FMR1/RGS4 double knockouts also demonstrated significant increases in total activity ($p < 0.01$), distance traveled ($p < 0.001$), and total rearing ($p < 0.01$) when compared to WT mice. Locomotor and exploratory behaviors did not differ between FMR1 knockout and FMR1/RGS4 double knockout mice ($p > 0.05$). Taken together, these results demonstrate (a) a transient increase in open field activity in the early time points of activity testing in RGS4 knockout mice compared to WT animals, and (b) as observed in the double knockout mice, knockout of RGS4 does not affect the sustained hyperactivity seen in FMR1 knockout mice.

RGS4 knockout does not affect novel object recognition in fragile X mice

FMR1 knockout mice have been reported to show a deficit in object recognition tasks (Restivo et al., 2005; Ventura et al., 2004). We tested adult WT ($n = 9$), RGS4 knockout ($n = 9$), FMR1 ($n = 10$) and FMR1/RGS4 double knockout ($n = 11$) mice in the novel object recognition

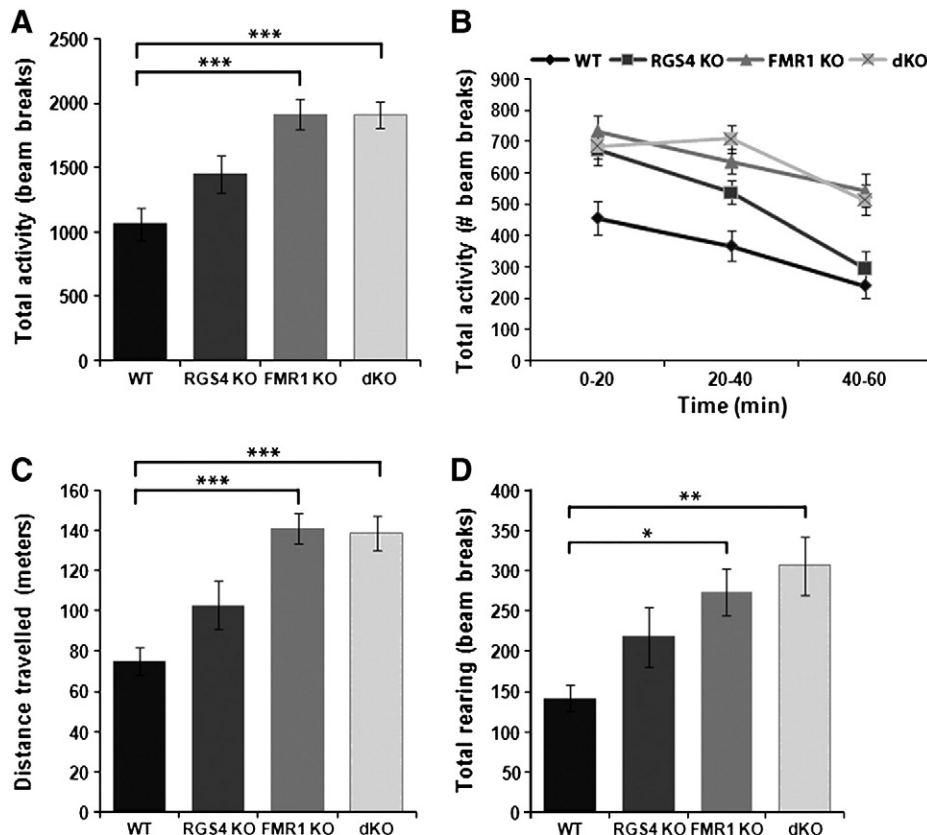


Fig. 7. Motor activity levels. Six week old wild-type ($n = 12$), FMR1 knockout ($n = 12$), RGS4 knockout ($n = 13$) and double knockout mice ($n = 12$) were monitored in the open field for 1 h. A, FMR1 knockout and FMR1/RGS4 double knockouts showed increased total activity compared to wild-type mice. Activity levels of double knockout mice were not different from FMR1 knockouts. B, Total activity of FMR1 knockout and double knockout was significantly increased at all time points compared to wild-type. RGS4 knockouts showed increased activity at 20 and 40 min ($p < 0.01$ and $p < 0.05$ respectively), when compared to wild-type animals; activity returned to wild-type levels in the final 20 min of testing. C, Total distance travelled was significantly greater for FMR1 knockout and double knockouts compared to wild-type mice. D, Total rearing activity was also increased in FMR1 knockout and double knockout compared to wild-type. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

task. When confronted with both familiar and novel objects, the FMR1 mice spent significantly less ($60 \pm 2.2\%$) of their total exploration time exploring the novel object, as compared to WT mice ($70 \pm 1.7\%$; $p < 0.05$). However, FMR1/RGS4 double knockout mice ($63 \pm 2.3\%$) did not differ significantly from WT or FMR1 knockouts ($p > 0.05$) in percentage of exploratory time spent with the novel object (Fig. 8).

Discussion

Regulator of G-protein signaling (RGS) proteins are important modulators of GPCR signaling. Increasing evidence of multiple GPCR signaling deficiencies in FMR1 knockout mice (Bear et al., 2004; Volk et al., 2007; Wang et al., 2008) prompted us to examine the role of RGS proteins in FMR1 mice. Within the RGS family, we focused on RGS4 because it is highly expressed in the nervous system, its distribution shows substantial overlap with FMRP, and because RGS4 is known to modulate GPCRs coupled to the Gq class of G-proteins.

Based on the rescue of the FMR1 audiogenic seizure phenotype in the FMR1/RGS4 double knockout mice, and on the inhibition of seizures in FMR1 mice with the GABA_B agonist baclofen, we previously proposed that RGS4 plays an important inhibitory role in GABA_B function in the brain (Pacey et al., 2009). The results of the current study demonstrating a robust up-regulation of GABA_B R1 protein in RGS4 deficient mice, and a down-regulation in FMR1 mice, lend further credence to the idea that RGS4 negatively regulates the GABA_B receptor. Based on studies showing that GABA_B R1a containing GABA_B receptors are expressed on glutamatergic nerve terminals and act to reduce glutamate release (Vigot et al., 2006; Guetg et al., 2009), we suggest that increased GABA_B function mediated by deletion of RGS4, or by administration of GABA_B agonists, may also dampen glutamate release thereby reducing over-active mGluR5 signaling in FXS.

Another potential explanation for how RGS4 knockout could alter fragile X phenotypes is that RGS4 mRNA may bind to and be regulated by FMRP. RGS5, a protein with high homology to RGS4, has been shown to be an mRNA substrate for FMRP (Miyashiro et al., 2003) suggesting that RGS4 might also be a substrate. However, in preliminary immunoprecipitation experiments using mouse brain tissue and an anti-FMRP antibody, we have been unable to detect a physical interaction between FMRP and RGS4 mRNA (Suppl. Fig. 3). Although we used established protocols for immunoprecipitation of FMRP/mRNA complexes and RT-PCR (Brown et al., 2001; Edbauer et al., 2010; Westmark and Malter, 2007), our results do not preclude the possibility that RGS4 mRNA binds FMRP under different experimental conditions and/or in a different cellular compartment (i.e. nucleus vs. cytoplasm). Using quantitative RT-PCR analysis we did not detect a difference in RGS4 mRNA expression in FMR1 mice

compared to wild-type. Unfortunately, due to the rapid turnover of RGS4 protein in the brain and the lack of specificity of currently available antibodies for RGS4 (see Suppl. Fig. 4), we have been unable to measure RGS4 protein levels, and therefore cannot rule out changes in the expression of RGS4 protein in FMR1 mice. Nonetheless, our findings suggest that the selective phenotypic rescue seen in FMR1/RGS4 double knockout mice is less likely caused by the normalization of abnormal RGS4 expression in FMR1 mice, and more likely to be due to the effects on downstream signaling pathways that are already abnormal in the absence of FMRP.

Individuals with FXS often display increased levels of social anxiety (Hall et al., 2009) and FMR1 knockout mice show similar alterations in sociability (Spencer et al., 2005; McNaughton et al., 2008; Liu and Smith, 2009). Our findings from two independent socialization tests – the tube test for social dominance and a conditioned place preference paradigm involving the scent of an unknown mouse – demonstrated social avoidance behavior in both FMR1 knockout and RGS4 knockout mice. To our knowledge, this is the first report of abnormal social behavior in RGS4 knockout mice. This finding could be taken as supportive of the suggested role of RGS4 in the susceptibility to schizophrenia (Prasad et al., 2005; Chowdari et al., 2008; Ding and Hegde, 2009; Prasad et al., 2010). Interestingly, the FMR1/RGS4 double knockout mice showed reversal of abnormal social interaction. This intriguing finding suggests that while loss of FMRP or RGS4 alone causes impaired sociability, the concurrent loss of both proteins restores normal social behavior in mice. The biological mechanism for this behavioral normalization is unclear; however, we speculate that it could be at least partially explained by the normalization of PSD-95 and GABA_A α1 expression observed in FMR1/RGS4 double knockout mice.

While RGS4/FMR1 double knockout mice showed reversal of some fragile X-related phenotypes, other phenotypes such as macroorchidism and hyperactivity, were unaffected by the loss of RGS4 (summarized in Table 1). The absence of effects on the latter phenotypes in the FMR1/RGS4 double knockout mice could be explained, in part, by the absence of overlap of the two genes in some brain regions and tissues. For example, the lack of reversal of testicular enlargement in the double knockouts can be explained by the reported absence of RGS4 in this tissue (Nomoto et al., 1997).

Within the CNS, the lack of effect on motor hyperactivity in the double knockouts is more difficult to explain because of the broadly distributed neuronal pathways involved in motor activity. RGS4 and FMRP are both highly expressed in some forebrain regions associated with motor activity such as the cerebral cortex (motor cortex) and the striatum. If hyperactivity was mediated by neurons in these regions, an effect on activity might have been anticipated. Although hyperactivity has traditionally been thought to be mediated by fronto-striatal circuitry, more recent evidence suggests that cerebellar deficits may also result in increased motor activity (Cherkasova and Hechtman, 2009; Martin et al., 2010). FMRP is highly expressed in the Purkinje cells of the cerebellum (Koekkoek et al., 2005; Zangenehpour et al., 2009) and studies have documented Purkinje cell loss and/or impairment of Purkinje cell-mediated neurotransmission in both FXS and in autism (Koekkoek et al., 2005; Huber, 2006; Fatemi et al.,

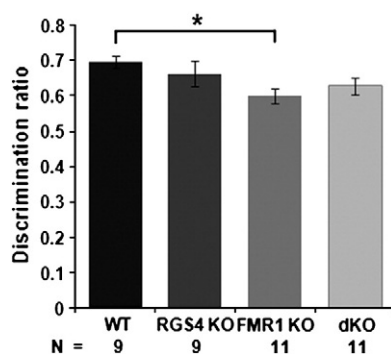


Fig. 8. Novel object recognition test. Adult male wild-type, RGS4 knockout, FMR1 knockout and FMR1/RGS4 double knockout mice were tested for novel object recognition. FMR1 knockout mice spent a significantly smaller percentage of exploratory time with the novel object when compared to wild-type mice. FMR1/RGS4 knockout mice did not differ from FMR1 knockouts in percentage of exploratory time spent with the novel object. * $p < 0.05$.

Table 1

Summary of effects of FMR1/RGS4 double knockout on Fragile X-related phenotypes.

Normalized	Not affected
Audiogenic seizures	Testes weight
Total body weight	Open field performance (hyperactivity)
PSD-95 expression (PND12, adult)	Novel object recognition
GABA _A receptor α1 subunit expression (PND12)	
Tube test for social dominance	
Conditioned place preference (scent aversion)	

2008). In contrast, RGS4 is virtually absent in the cerebellum (Gold et al., 1997; see also the RGS4 gene distribution depicted in the Allen Brain Atlas, <http://www.brain-map.org/>). Thus, based on the absence of an effect on motor activity in the FMR1/RGS4 double knockout mice and the lack of expression of RGS4 in the cerebellum, together with previous imaging and anatomical findings in both humans and mice, we suggest that cerebellar abnormalities might play a role in the hyperactivity seen in FXS.

In summary, we have provided evidence that absence of the regulatory protein RGS4 rescues several fragile-X related phenotypes, including increased body weight, abnormal synaptic protein expression and inhibition of social behaviors, in FMR1 knockout mice. The biochemical mechanism(s) that explain these results are likely multifaceted and complicated, involving changes in multiple signaling pathways and/or receptors. Further investigation of the role of RGS4 in the brain may provide a better understanding of how loss of this regulatory protein can compensate for the absence of FMRP. Our findings also suggest that inhibitors of RGS4 may have therapeutic value in treating FXS and provide further motivation for searching for novel selective inhibitors of RGS proteins (Blazer and Neubig, 2009; Roof et al., 2009).

Experimental methods

Animals

All animal experiments were carried out in accordance with the guidelines set by the Canadian Council on Animal Care and were approved by the University of Toronto Animal Care Committee. The *Rgs4^{tm1Dgen}/J* knockout mouse strain (Cifelli et al., 2008) was backcrossed seven generations onto the C57BL/6 background. FMR1 knockout mice (backcrossed >10 generations on the C57BL/6 background) were generously provided by Dr. William Greenough, University of Illinois, and bred at the University of Toronto. FMR1/RGS4 double knockout mice were created and genotyped as previously described by Pacey et al (2009).

Quantitative RT-PCR

Hippocampus and cerebral cortex were dissected from wild-type and FMR1 knockout mice at postnatal day (PND) 12, PND 30 and adult mice (2–4 months old) and RGS4 mRNA levels were measured by quantitative RT-PCR as described in Cifelli et al. (2008). Briefly, total RNA was extracted from tissues using TRIzol reagent (Invitrogen Life Technologies). All quantitative RT-PCR was performed using an ABI Prism 7900 HT (Applied Biosystems) using the Sybr Green detection system. Two micrograms of total RNA was reverse transcribed with random hexamer primers using the Superscript II kit (Invitrogen Life Technologies) following the manufacturer's protocols. cDNA was diluted to a final volume of 280 μ L. Two microliters of the RT reaction mixture was subsequently used as a template for real time PCR quantification. Each cDNA sample was evaluated for RGS4 (forward 5' GGGCTGAATCGTTGGAAAAC 3'; reverse 5' ATCCGACTTCAGGAAAGCTTT 3') and the housekeeping gene GAPDH (forward, 5' TTCACCACCATGGAGAAGG 3'; reverse, 5' CTCGTGGTTCACCCATC 3') to serve as a normalizing control in independent wells. Data obtained from the PCR reaction were analyzed using the comparative CT method (User Bulletin No. 2, Perkin Elmer Life Sciences). Data represent the relative mRNA levels for RGS4 in these tissues. N values were as follows: PND 12 cortex and hippocampus (WT = 8, KO = 7); PND 30 cortex and hippocampus (WT = 8, KO = 7); adult cortex (WT = 6, KO = 8); and adult hippocampus (WT = 8, KO = 7).

Western blotting

Postnatal day 12 or adult (2–4 months old) WT, FMR1 knockout, RGS4 knockout and FMR1/RGS4 double knockout mice were euthanized by cervical dislocation and the brains were removed and placed

on ice. The cerebellum was removed and one half of the forebrain was homogenized in ice cold 50 mM Tris-HCl, 1% SDS, pH 7.4 supplemented with protease inhibitor cocktail (Sigma) using a glass/teflon homogenizer. The protein concentration was determined using the BCA assay (Sigma). Equal amounts of protein (6–30 μ g depending on the abundance of the target protein) were loaded onto a 10% polyacrylamide-SDS gel and transferred onto a nitrocellulose membrane after electrophoresis. The membranes were blocked in 5% milk overnight and probed with the anti-PSD-95 antibody (clone K28/43; 1:200,000; NeuroMab, University of California, Davis/NIH), anti-GABA_A α 1 subunit antibody (1:1000; Upstate), anti-GABA_BR1 antibody (clone NR3A/49; 1:250; NeuroMab, University of California, Davis/NIH), anti-GABA_BR2 antibody (clone N81/2; 1:750; NeuroMab, University of California, Davis/NIH) and anti-GAPDH antibody (1:40,000–1:100,000; Sigma) and a donkey anti-mouse (Jackson Labs) or goat anti-rabbit (Pierce) HRP-conjugated secondary antibody. The immunoreactive proteins were visualized using the FluorChem™ Multimage Light Cabinet (Alpha Innotech). Densitometric analysis was carried out using the AlphaEaseFC software (Alpha Innotech). The intensity of the band of interest was normalized relative to the GAPDH band intensity. Protein expression in FMR1, RGS4 and double knockout animals is presented as a percentage of wild-type expression levels. A one-way ANOVA with Tukey's post-hoc analysis was used to determine statistical significance.

Tube test for social dominance

To examine social behaviors, adult (8–9 weeks old) male mice were tested using the tube test for social dominance (Lindzey et al., 1961; Spencer et al., 2005). Each match involved two mice of different genotypes that were not housed together. One mouse was placed into each end of a transparent PVC tube (2.5 cm inner diameter, 30.5 cm length) and the mice were released simultaneously. The match ended when one mouse completely retreated from the tube with the mouse remaining in the tube being deemed the "winner". The number of wins for each genotype was tallied and a chi-square analysis was used to determine whether the scores were significantly different from the 50:50 win/loss outcome expected by chance. Each animal was tested three times each day against three different animals of opposing genotypes, over a maximum of three days. Testing was carried out between 1:00 and 5:00 pm.

Conditioned place preference

Conditioned place preference has traditionally been used to measure drug reward or aversion; however, this paradigm can be adapted to measure most appetitive or aversive stimuli (for review see Tzschentke, 1998, 2007). We have adapted this test to measure social behavior in mice by testing for a preferential or aversive response to the scent of another animal. The testing apparatus (Place Preference Chamber, Medassociates Inc) consisted of a box with overall inside dimensions of 46.5 cm \times 12.7 cm \times 12.7 cm separated into two choice compartments (16.8 cm long) and a centre compartment (7.2 cm long). One choice compartment was all black with a stainless steel grid rod floor. The other was all white with a stainless steel mesh floor. Ceiling lights were placed on the lid of both chambers to ensure adequate lighting.

Male adult (2–4 months old) WT, FMR1 knockout, RGS4 knockout and FMR1/RGS4 double knockout mice (n = 8 per genotype) were used in this paradigm. Control animals (age- and sex-matched wild-type C57BL/6 mice) were allowed to freely explore the conditioning compartment for 10 min after which the control animals were removed and the conditioning groups were placed in the compartment for 5 min. The animals were returned to their home cage following conditioning. Mice were counterbalanced to each compartment in order to ensure that no baseline compartment preference existed such that, half the animals experienced a test stimulus on the dark side and the other half

experienced the stimulus on the white side. Conditioning occurred once daily for four consecutive days. On the fifth day (“test day”), the partition between the two compartments was removed and the animals were allowed to freely explore either the scent paired side or the neutral side for 10 min. A change score was calculated for each mouse by subtracting the time spent in the stimulus side from the time spent in the neutral side. A positive score denotes a preference for the stimulus, whereas a negative score indicates an aversion. In order to make the test apparatus as similar as possible to the training environment, animals were tested with the scent of the control wild-type present.

Open field testing

Open field behavior was tested in male WT, FMR1 knockout, RGS4 knockout and FMR1/RGS4 double knockout mice at 6 weeks of age. An automated movement detection system (Activity Monitors, Linton Instrumentation, UK) was used to measure motor behavior as previously described (Jugloff et al., 2008). Briefly, the apparatus consisted of a plexiglass box (24×45 cm) surrounded by a frame (26×47 cm) from which 24 infrared beams emerge to form a grid across each of two levels. As the mouse moves, a beam is broken and an activity count is registered. The software (Amonlite, MJS Technology, UK) measures several behavioral parameters, including total activity, distance traveled and total rearing. Mice were placed individually into the open field apparatus and behavior was assessed in 5 minute epochs for a total of 1 h per animal. Experiments were carried out between 9:00 a.m. and 2:00 p.m. Each animal was tested in the open field once. A one-way ANOVA followed by Tukey's post hoc analysis was used to determine statistical significance. For comparisons of activity levels over time, data was analyzed using a two-way repeated measures ANOVA with post hoc Bonferroni test comparing all genotypes.

Novel object recognition task

Adult (2–4 month old) male WT, FMR1 knockout, RGS4 knockout and RGS4/FMR1 double knockout mice were habituated to the testing cage (a transparent plexiglass mouse cage of dimensions 28×17×14 cm) for 60 min on each of the two days leading up to the experiment. On the third day, animals were placed individually into the cage and allowed to roam around for 5 min after which two identical objects (glass scintillation tubes filled with sand) were placed into adjacent corners of the cage at a distance of approximately 4 cm from the cage wall. The mouse was allowed to explore for 10 min during which the amount of time spent exploring each object was recorded. After 10 min, both objects were removed and the mouse remained in the empty cage for 3 min. After 3 min, a third identical object (glass scintillation tube with sand) and a novel object (metal padlock) were placed in the same locations as the original objects. The amount of time the mouse spent exploring each object was scored for 10 min. Exploratory behavior was defined as any behavior directed at the object including sniffing (within ~5 cm), touching, chewing, pawing (directed touch), rearing or standing on the object. Indirect/unintentional contact with the object was not considered an exploratory behavior. The amount of time spent exploring the novel object was normalized to the total amount of time spent exploring both objects and expressed as a discrimination ratio. The familiar and novel objects were randomly placed on the left or right with each experiment. Experiments were scored by an observer blind to the genotype of the mice. A one-way ANOVA with post-hoc Tukey's test was used to determine statistical significance.

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