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ACCELERATED COMMUNICATION

Increased $GABA_B$ Receptor-Mediated Signaling Reduces the Susceptibility of Fragile X Knockout Mice to Audiogenic Seizures

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ABSTRACT

Mice lacking the gene encoding fragile X mental retardation protein (FMR1) are susceptible to audiogenic seizures, and antagonists of the group I metabotropic glutamate receptors (mGluRs) have been shown to block seizures in FMR1 knockout mice. We investigated whether the G-protein-inhibitory activity of the regulator of G-protein signaling protein, RGS4, could also alter the susceptibility to audiogenic seizures in FMR1 mice. We were surprised to find that male FMR1/RGS4 double-knockout mice showed reduced susceptibility to audiogenic seizures compared with age-matched FMR1 mice. These data raised the intriguing possibility that loss of RGS4 increased signaling through another G-protein pathway that reduces seizure susceptibility in FMR1 mice. Indeed, administration of the $GABA_B$ receptor agonist baclofen to FMR1 mice inhibited seizures, whereas the $GABA_B$ receptor antagonist (3-aminopropyl)(cyclohexylmethyl)phosphinic acid (CGP 46381) increased seizure incidence in double-knockout mice but not in wild-type mice. Finally, audiogenic seizures could be induced in wild-type mice by coadministering CGP 46381 and the mGluR5-positive allosteric modulator 3-cyano-*N*-(1,2 diphenyl-1H-pyrazol-5-yl) benzamide. These data show for the first time that $GABA_B$ receptor-mediated signaling antagonizes the seizure-promoting effects of the mGluRs in FMR1 knockout mice and point to the potential therapeutic benefit of $GABA_B$ agonists for the treatment of fragile X syndrome.

Fragile X syndrome results from a mutation in the X-linked FMR1 gene leading to the absence of the gene product fragile X mental retardation protein (FMRP). Persons with fragile X exhibit a spectrum of abnormalities including mild to moderate mental retardation, impaired learning and memory, hyperactivity, and anxiety (O'Donnell and Warren, 2002; Bagni and Greenough, 2005). Autistic-like behaviors and seizures are present in approximately 20% of such persons (Wisniewski et al., 1991; Bailey et al., 2008). The metabotropic glutamate receptor (mGluR) theory of fragile X posits that FMRP regulates the translation of specific mRNAs expressed in response to group I mGluR activation. In the absence of FMRP, group I mGluR signaling is enhanced, leading to several neurological alterations associated with fragile X syndrome (Bear et al., 2004). Recent evidence indicates that perturbations in cellular signaling in fragile X extend to GABA-gated anion channels (Centonze et al., 2008; Chang et al., 2008; Curia et al., 2008) and to other non-mGluR GPCRs, including dopamine receptors and muscarinic acetylcholine receptors (Volk et al., 2007; Wang et al., 2008).

The FMR1 knockout mouse exhibits many characteristics that mimic fragile X in humans and is widely used to study fragile X syndrome. One of the most robust and reproducible phenotypes in the FMR1 knockout mouse is susceptibility to

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ABBREVIATIONS: FMRP, fragile X mental retardation protein; GPCR, G-protein-coupled receptors; K_{IR}, inward-rectifying potassium; mGluR, metabotropic glutamate receptor; CGP 46381, (3-aminopropyl)(cyclohexylmethyl)phosphinic acid; RGS, regulator of G-protein signaling; CDPPB, 3-cyano-*N*-(1,2 diphenyl-1H-pyrazol-5-yl) benzamide; PCR, polymerase chain reaction; bp, base pair(s); RT-PCR, reverse-transcription polymerase chain reaction; KO, knockout.

audiogenic seizures (Musumeci et al., 2000, 2007; Chen and Toth, 2001; Yan et al., 2004, 2005; Dölen et al., 2007). GPCRs, including the group I mGluR mGluR5 (Yan et al., 2005; Dölen et al., 2007) and GABA$_B$ receptors (Faingold, 2002), have been implicated in the development of audiogenic seizures in rats and mice, and the mGluR5 antagonist MPEP has been shown to reduce seizures in FMR1 mice (Yan et al., 2005) and to reduce the dendritic spine abnormalities in cultured neurons from FMR1 mice (de Vrij et al., 2008).

Regulator of G-protein signaling (RGS) proteins are GTPase-activating proteins for heterotrimeric G-protein α subunits that can modulate GPCR signaling (Abramow-Newerly et al., 2006; Blazer and Neubig, 2009). RGS proteins are important regulators of GPCR signaling, and alterations in RGS protein expression have been suggested to play a role in a number of disease states (Muma et al., 2003; Riddle et al., 2005; Liu et al., 2006). RGS4 has been shown to be a potent inhibitor of both G$_q$- and G$_{i/o}$-coupled pathways; however, the specific GPCR pathways that it regulates in vivo are not known. RGS4 is highly expressed in the developing and adult brain (Nomoto et al., 1997; Ingi and Aoki, 2002), in which it inhibits the signaling of group I mGluRs (Saugstad et al., 1998). More recently, RGS4 has been shown to associate with GABA$_B$ receptors and inward-rectifying K$^+$ channels (K$_{IR}$) (Fowler et al., 2007), suggesting that it may also regulate GABA$_B$-mediated signaling. In the FMR1 knockout mouse, RGS4 mRNA was shown to be decreased in hippocampus and cortex during early postnatal development (Tervonen et al., 2005), indicating a possible role for RGS4 in the pathogenesis of fragile X syndrome.

The objective of the present study was to examine the role of RGS4 in fragile X syndrome. We crossed FMR1 knockout mice with RGS4 knockout mice to produce FMR1/RGS4 double knockouts. Because removing RGS4 may be expected to enhance mGluR signaling, we anticipated observing exacerbated symptoms of fragile X in double-knockout mice. Instead, we demonstrate that the absence of RGS4 expression rescues audiogenic seizure susceptibility in FMR1 knockout mice and that this effect is partly mediated by increased signaling through GABA$_B$ receptors. These findings suggest that audiogenic seizures in FMR1 knockout mice may be caused by an imbalance in mGluR and GABA-mediated signaling.

Materials and Methods

Animals. All animal experiments were carried out in accordance with the guidelines set out 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 (described by Cifelli et al., 2008) was backcrossed seven generations onto the C57BL/6 background. FMR1 knockout mice on the C57/Bl6 background were generously provided by Dr. William Greenough (University of Illinois, Urbana, IL) and bred at the University of Toronto. FMR1/RGS4 double knockout mice were created by breeding RGS4 knockout male mice with FMR1 knockout female mice. The resultant female (FMR1 heterozygote, RGS4 heterozygote) and male (FMR1 knockout, RGS4 heterozygote) offspring were crossed, and some F2 generation offspring were used for seizure experiments. FMR1/RGS4 double knockout offspring of the F2 generation were mated to produce pure double knockout lines.

Genotyping. FMR1 genotyping was based on the presence or absence of the wild-type or knockout FMR1 allele as described previously (Dölen et al., 2007). For the wild-type allele, primers S1 (5′-GTG GTT AGC TAA AGT GAG GAT GAT-3′) and S2 (5′-CAG GTT TGT TGG GAT TAA CAG ATC-3′) were used. For the FMR1 knockout allele, primers M2 (5′-ATC TAG TCA TGC TAT GGA TAT CAG C-3′) and N2 (5′-GTG GGC TCT ATG GCT TCT GAG G-3′) were used. The following PCR conditions were used: 95°C for 5 min; 34 PCR cycles of 30 s at 95°C, 30 s at 61°C, and 1 min at 72°C; and 10 min at 72°C. Wild-type and mutant mouse PCR reactions were run separately. The reaction products were combined and separated on a 1.5% agarose gel. The wild-type and knockout alleles produced bands of 528 and 800 bp, respectively.

Screening for the RGS4 gene was based on the presence or absence of the wild-type or knockout alleles. For the wild-type allele, GSET (5′ CCA TCT TGA CCC AAA TCT GGC TCA G 3′) and GSE1 (5′ GGA CAT GAA ACA TCG GCT GGG GTT C 3′) were used. For the knockout allele, GSET and NeoT (5′ GGG CCA GCT CAT TCC TCC CAC TCA T 3′) were used. The following PCR program was used: 5 min at 95°C; 34 PCR cycles of 30 s at 95°C, 30 s at 70°C, and 1 min at 72°C; and 10 min at 72°C. Wild-type and knockout PCR reactions were run separately. The wild-type and knockout alleles produced bands of 226 and 484 bp, respectively.

RT-PCR. Total RNA was isolated from mouse forebrain using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Two micrograms of total RNA was reverse-transcribed with random nonomers (Sigma, St. Louis, MO) using the Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) as described by the manufacturer. PCR was performed using RGS4 cDNA-specific forward (5′-GCC AAG AAG AAG TCA AGA AAT GGG C-3′) and reverse (5′-TGG CTC CTT TCT GCT TCT CTG CC-3′) primers. The following PCR reaction was run: 95°C for 10 min; and 30 PCR cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min. The reaction products were separated on a 1.5% agarose gel. The presence of the RGS4 cDNA was indicated by a 420-bp band.

Western Blotting. Adult wild-type, FMR1 knockout, and FMR1/RGS4 double-knockout mice were euthanized with an overdose of ketamine/xylazine, and the brains were removed and placed on ice. One half of the forebrain was homogenized in ice-cold 50 mM Tris and 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). Twenty micrograms of protein per sample was 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 2F5-1 anti-FMRP antibody (1:1000; gift of Jennifer Darnell, The Rockefeller University, New York, NY) and a donkey anti-mouse horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The immunoreactive proteins were visualized using the FluorChem MultiImage Light Cabinet (Alpha Innotech, San Leandro, CA).

Audiogenic Seizure Testing and Drug Injections. For audiogenic seizure testing, the apparatus consisted of a Plexiglas mouse cage (28 × 17 × 14 cm) with a 135-dB sound source (Piezo siren, Electrosonic; Piezo Technologies, Indianapolis, IN) attached to the lid and extending 5 cm down into the cage. Mice (27–30 days old) were placed individually into the testing apparatus and were allowed to explore for 2 min, after which the bell was rung for 2 min. Seizure activity was observed and scored using a seizure severity score as follows: wild running = 1; clonic seizure = 2; tonic seizure = 3; status epilepticus/respiratory arrest/death = 4 (Musumeci et al., 2000). Animals were considered to have had a seizure if the seizure severity score was greater than 1. Animals were tested only once. Seizure testing was carried out between 1:00 PM and 6:00 PM.

For drug injection studies, an intraperitoneal injection of drug or vehicle (0.1 ml/10 g body weight) was administered 30 or 45 min before seizure testing. The drug doses and vehicles are as follows: 2.0 mg/kg (*R*)-baclofen (Sigma/RBI, Natick, MA) in saline; 60 mg/kg CPG 46381 (Tocris Bioscience, Ellisville, MO) in saline; and 2.5 mg/kg 3-cyano-*N*-(1,2 diphenyl-1*H*-pyrazol-5-yl) benzamide (CDPPB; Tocris

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Bioscience) in 50% dimethyl sulfoxide/50% sterile saline. Fisher's exact test was used for statistical analysis of seizure susceptibility data.

Results

FMR1/RGS4 Double-Knockout Mice Do Not Express FMRP or RGS4. FMR1/RGS4 double-knockout mice were created by crossing female FMR1 knockout and male RGS4 knockout mice. The resulting offspring were mated, and the F2 generation was genotyped for FMR1 and RGS4 (Fig. 1, A and B). FMR1/RGS4 double knockouts from the F2 generation were subsequently mated to produce pure double-knockout lines. Western blots of adult forebrain tissue demonstrated the absence of FMRP expression in FMR1/RGS4 double KO mice (Fig. 1C). Because RGS4 protein is difficult to detect on Western blots, RT-PCR was used to probe for RGS4 mRNA expression. FMR1/RGS4 double-knockout mice did not express RGS4 mRNA (Fig. 1D). These results verify that the double-knockout mice did not express FMRP or RGS4.

RGS4 Knockout Rescues Audiogenic Seizures in FMR1 Knockout Mice. Postnatal day 27 to 30 mice were exposed to a 135-db alarm for 2 min, and seizure susceptibility was evaluated as described previously (Musumeci et al., 2000). In total, 53% of FMR1 knockout mice exhibited sound-induced seizures compared with 4% of wild-type animals (Fig. 2A, $p < 0.001$). FMR1/RGS4 double-knockout mice displayed a 71% reduction in seizure incidence compared with FMR1 knockout mice ($p < 0.01$). The incidence of seizures in FMR1/RGS4 double-knockout mice was not statistically different from that of wild-type animals ($p > 0.05$). It is noteworthy that we also observed a trend toward decreased seizure susceptibility in male FMR1 knockout mice heterozygous for RGS4 (Fig. 2A, $p = 0.07$).

Because FMR1 is an X-linked gene and the possible genotype combinations differ based on gender, we also analyzed the seizure data separately for male and female mice (Fig. 2, B and C, respectively). Male FMR1/RGS4 double-knockout mice displayed a significant 88% reduction in seizure incidence compared with the male FMR1 mice (Fig. 2B, $p < 0.01$). The incidence of seizures was reduced by 46% in female FMR1/RGS4 double knockouts compared with female FMR1 mice, although this difference did not reach statistical significance (Fig. 2C, $p > 0.05$). The reason for this differential effect is unclear but may be accounted for by hormonal dif-

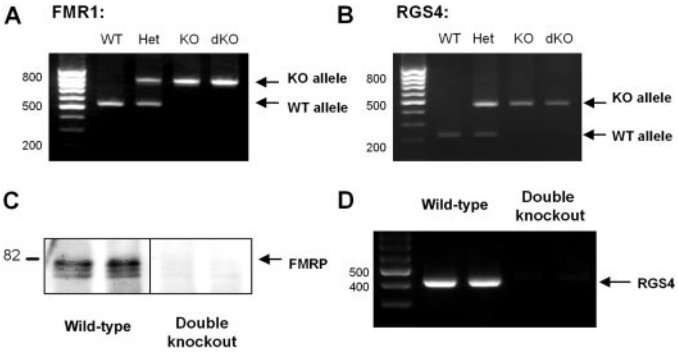

Fig. 1. FMR1/RGS4 double-knockout mice do not express FMRP or RGS4. In A and B, genotyping for FMR1 and RGS4 was carried out using PCR with primers specific to the wild-type and knockout alleles of each gene. In A, bands at 534 and 800 bp indicate the presence of the FMR1 wild-type and knockout alleles, respectively. B, bands at 226 and 484 bp indicate the RGS4 wild-type and knockout alleles, respectively. C, Western blot analysis using an anti-FMRP antibody demonstrating expression of FMRP (~80 kDa) in the forebrain of wild-type but not FMR1/RGS4 double-knockout mice. D, RT-PCR analysis demonstrates the absence of RGS4 mRNA expression (446 bp band) in the forebrain of FMR1/RGS4 double-knockout mice. Het, heterozygote; dKO, FMR1/RGS4 double knockout.

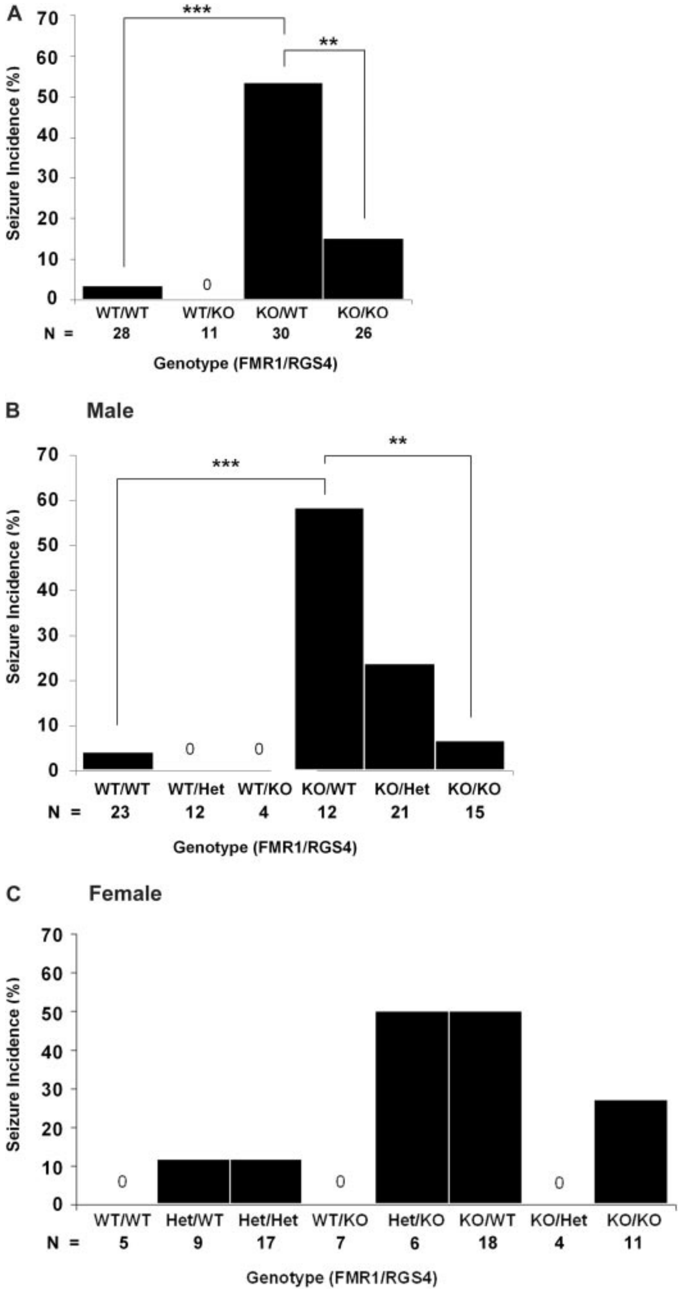

Fig. 2. RGS4 knockout reduces audiogenic seizures in the FMR1 knockout strain. Mice were tested for audiogenic seizures at postnatal day 27 to 30. A, FMR1 knockout mice were more susceptible to audiogenic seizures than wild-type animals (***, $p < 0.001$). FMR1/RGS4 double-knockout mice showed a statistically significant reduction in audiogenic seizures compared with FMR1 knockout mice (**, $p < 0.01$). The incidence of seizure activity in double-knockout mice was not significantly different from that of wild-type ($p > 0.05$). B, male FMR1/RGS4 double-knockout mice showed a statistically significant decrease in seizure activity compared with male FMR1 knockouts (**, $p < 0.01$). C, female FMR1/RGS4 double-knockout mice showed a trend toward reduced susceptibility to audiogenic seizures compared with FMR1 knockout female mice; however, this reduction did not reach statistical significance ($p > 0.05$).

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ferences in female versus male mice or differences attributed to the hemizygous (XY) versus homozygous (XX) genotype. To our knowledge there is no evidence to suggest sex differences in RGS4 expression or function, but further study is needed to more closely examine this issue.

RGS4 heterozygous and RGS4 knockout male mice (Fig. 2B; WT/Het and WT/KO, respectively) and RGS4 knockout female mice (Fig. 2C; WT/KO) did not exhibit seizures. FMR1 heterozygous female mice (Fig. 2C; Het/WT, Het/Het, and Het/KO) showed some seizure activity, irrespective of their RGS4 genotype, but this was not significantly different from wild-type levels. Together, these results demonstrate that ablating one or both copies of the RGS4 gene reduces seizure susceptibility in fragile X knockout mice.

The GABA$_B$ Agonist Baclofen Reduces Audiogenic Seizures in FMR1 KO Mice. To test for a role of GABA$_B$ receptors in audiogenic seizure susceptibility, 27- to 30-day-old FMR1 knockout mice (19 male and 31 female) were treated with the GABA$_B$ agonist (*R*)-baclofen (1.0 or 2.0 mg/kg i.p.) administered 45 min before seizure testing. Treatment with 1.0 and 2.0 mg/kg (*R*)-baclofen produced a 67% ($p < 0.05$) and 79% ($p < 0.01$) decrease, respectively, in seizure incidence (Fig. 3) compared with vehicle controls. When analyzed separately, both male and female FMR1 knockout mice showed statistically significant decreases ($p < 0.05$) in seizure activity at both doses (data not shown). These results demonstrate that stimulating GABA$_B$-mediated signaling rescues seizures in FMR1 knockout mice.

Treatment with a GABA$_B$ Antagonist Induces Seizures in FMR1/RGS4 Double-Knockout Mice but Not in Wild-Type Mice. Having demonstrated an anticonvulsant effect of the GABA$_B$ agonist baclofen on audiogenic seizures in FMR1 knockout mice, we sought to determine whether decreasing GABA$_B$ receptor-mediated signaling could induce seizures in wild-type mice. At 27 to 30 days of age, male wild-type mice were given an intraperitoneal injection of the GABA$_B$ antagonist CGP 46381 (60 mg/kg) 45 min before seizure testing. This treatment did not induce audiogenic seizures in wild-type or RGS4 knockout mice (Fig. 4). Although the dose of CGP 46381 used was approximately 10-fold higher than the IC$_{50}$ value of this drug (Olpe et al., 1993), it is conceivable that a higher dose of CGP 46381 could have reduced seizure incidence. Nevertheless, this result indicates that reducing GABA$_B$-mediated signaling alone is insufficient to induce seizures in wild-type mice. However, when the same 60 mg/kg dose of CGP 46381 was tested on FMR1/RGS4 double-knockout mice, seizures were observed in 86% of double-knockout male mice ($p < 0.05$); this represented approximately a 5-fold increase in seizure incidence over vehicle controls (Fig. 4). This finding suggests that genetically eliminating expression of RGS4 rescues the audiogenic seizure phenotype in FMR1 knockout mice by increasing signaling through GABA$_B$ receptors.

Coadministration of a GABA$_B$ Antagonist and an mGluR5-Positive Allosteric Modulator Induces Audiogenic Seizures in Wild-Type Mice. We hypothesize that the GABA$_B$ antagonist CGP 46381 can induce seizures in FMR1/RGS4 double-knockout mice because they already have increased mGluR signaling compared with wild-type mice. To test whether increased mGluR signaling coupled with reduced GABA$_B$ receptor mediated signaling could induce seizures in wild-type mice, male wild-type mice were administered CGP 46381 together with the mGluR5-positive allosteric modulator CDPPB at doses that did not induce seizures when administered alone. At high doses (greater than 10 mg/kg), CDPPB induced seizure activity in 27- to 30-day-old wild-type mice (result not shown). However, a dose of 2.5 mg/kg CDPPB administered intraperitoneally 30 min before testing did not induce seizure activity in these animals (Fig. 5). Likewise, a 60 mg/kg dose of CGP 46381 administered intraperitoneally 30 min before testing did not elicit seizures in wild-type mice (Fig. 5). However, when 2.5 mg/kg CDPPB was administered in combination with 60 mg/kg CGP 46381, 75% of wild-type mice exhibited seizure activity (Fig. 5). This result provides additional evidence that an imbalance between group I mGluR and GABA$_B$ receptor signaling promotes seizures.

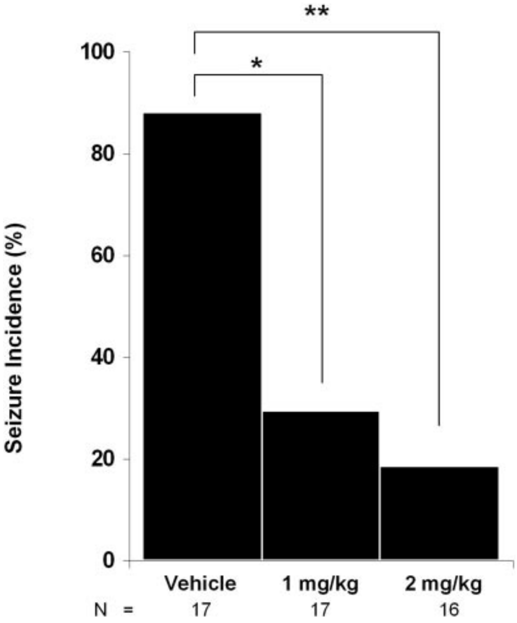

Fig. 3. Baclofen blocks seizures in FMR1 knockout mice. Mice (27–30 days old) were treated with 1 or 2 mg/kg (*R*)-baclofen. Baclofen significantly reduced audiogenic seizure incidence in FMR1 mice compared with vehicle controls (*, $p < 0.05$; **, $p < 0.01$).

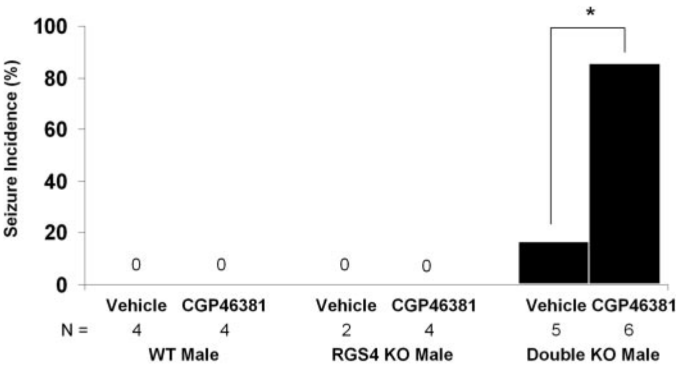

Fig. 4. The GABA$_B$ antagonist CGP 46381 induces audiogenic seizures in FMR1/RGS4 double-knockout but not wild-type mice. Male wild-type, RGS4 knockout and FMR1/RGS4 double-knockout mice were tested for seizure susceptibility at postnatal day 27 to 30. Treatment with 60 mg/kg of the GABA$_B$ antagonist CGP 46381 resulted in a significant increase in seizures in double-knockout male mice (*, $p < 0.05$). No seizure activity was observed in wild-type or RGS4 knockout animals with or without drug treatment.

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Discussion

We evaluated audiogenic seizure susceptibility, a well established phenotype of FMR1 knockout mice (Musumeci et al., 2000; Yan et al., 2004). Because RGS4 overexpression can attenuate signaling through mGluR5 (Saugstad et al., 1998), we postulated that FMR1/RGS4 double-knockout mice may show increased seizures compared with FMR1 single knockouts. Instead, a dramatic reduction in audiogenic seizures was observed in the double-knockout mice. This result suggested that in addition to mGluRs, other GPCR-dependent mechanisms may regulate the sensitivity of FMR1 mice to audiogenic seizures.

The complete list of specific pathways regulated by RGS4 in vivo is not known; however, our recent work shows that $G_{i/o}$-coupled signaling to inward rectifying potassium (K_{IR}) channels is markedly increased in the hearts of rgs4-null mice (Cifelli et al., 2008). It is possible that enhanced signaling through an analogous pathway in neurons (i.e., $GABA_B$ receptor activation of K_{IR} channels) may explain the observed decrease in susceptibility to audiogenic seizures. Indeed, alterations in GABA-mediated signaling have been shown to influence the development of audiogenic seizures (Caspary et al., 1984; Faingold et al., 1994). In support of a role for a protective effect of GABA signaling in the prevention of fragile X seizures, we observed reduced seizures in FMR1 knockout mice after treatment with the $GABA_B$ receptor agonist (*R*)-baclofen. $GABA_B$ receptors are $G\alpha_{i/o}$-coupled receptors; activation of $GABA_B$ receptors leads to reduced cAMP production and stimulates the opening of K_{IR} channels, leading to hyperpolarization and increased membrane potential (Jacobson et al., 2007; Labouèbe et al., 2007; Ulrich and Bettler, 2007). Mounting evidence suggests that, in addition to its GAP activity, RGS4 may be selectively targeted to different GPCR-K_{IR} signaling complexes in different cell types (Jaén and Doupnik, 2006). RGS4 has been shown to interact with $GABA_B$ receptors and K_{IR} channels (Fowler et al., 2007), suggesting the possibility that $GABA_B$ receptors are regulated by RGS4.

In the thalamus, mGluR activation enhances, whereas $GABA_B$ receptor activation suppresses, auditory signals necessary for sound detection (Schwarz et al., 2000). Several brain regions implicated in the development and progression of auditory seizures in rodents, including the cochlea, inferior and superior colliculus, and periaqueductal gray, express mGluR5, $GABA_B$ receptors, and/or RGS4 (Romano et al., 1995; Gold et al., 1997; Margeta-Mitrovic et al., 1999; Ross and Coleman, 2000; Friedland et al., 2006; Maison et al., 2009). We propose that in the auditory pathways involved in seizure induction and progression, auditory signals are balanced by mGluR (activating) and $GABA_B$ receptor (suppressing) signaling (Fig. 6). In wild-type animals, a balance between mGluR and $GABA_B$ receptor signaling is maintained, and loud sounds do not induce seizure activity. However, in

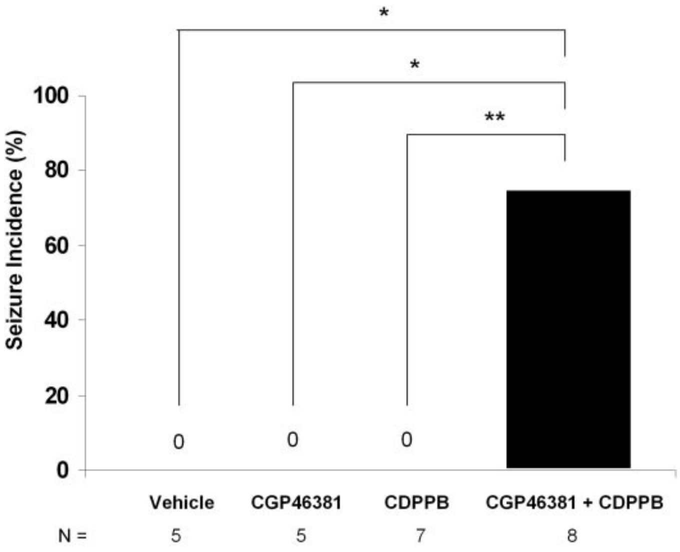

Fig. 5. Cotreatment with an mGluR5-positive modulator and a $GABA_B$ antagonist induces seizures in wild-type mice. Treatment with 2.5 mg/kg CDPPB (a mGluR5-positive allosteric modulator) or 60 mg/kg CGP 46381 (a $GABA_B$ antagonist) alone did not induce seizures in male wild-type mice. However, the combined treatment of 2.5 mg/kg CDPPB with 60 mg/kg CGP 46381 induced seizures in wild-type animals ($*$, $p < 0.05$; $**$, $p < 0.01$).

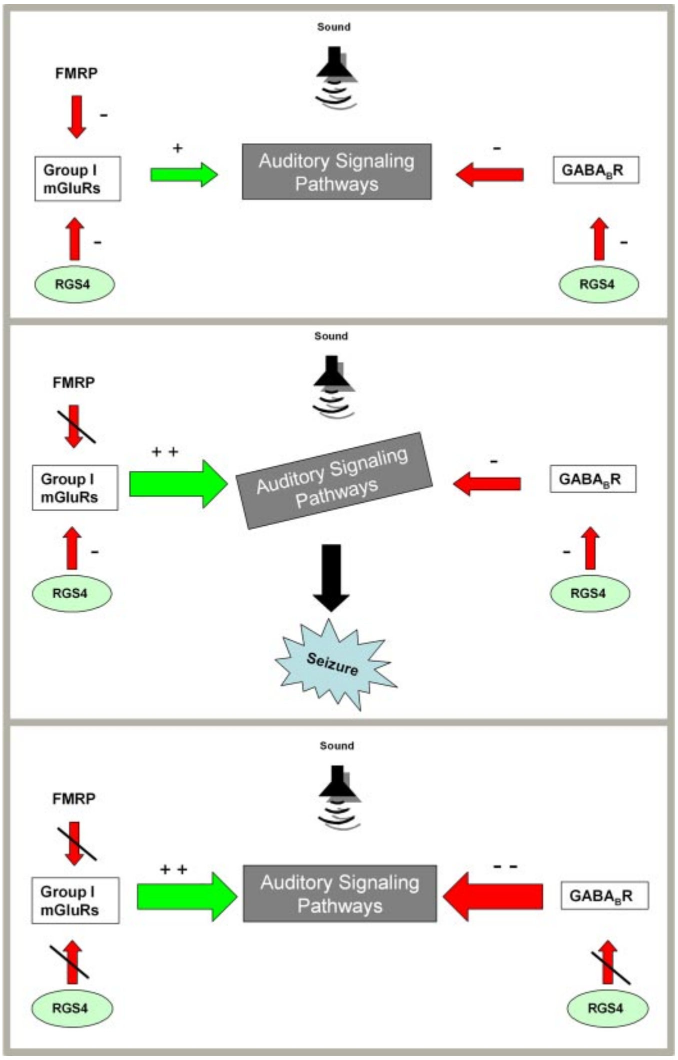

Fig. 6. Proposed model depicting group I mGluR and $GABA_B$ receptor signaling in audiogenic seizures. Top, in wild-type animals, a balance between group I mGluR and $GABA_B$ receptor-mediated signaling in auditory pathways prevents seizures. Middle, in FMR1 knockout mice, mGluR signaling is enhanced, altering the balance in favor of increased auditory signaling and giving rise to seizures. Bottom, in FMR1/RGS4 double knockout mice, the absence of RGS4 expression enhances $GABA_B$ receptor signaling, thus restoring the balance in auditory signaling pathways and reducing or preventing the seizures.

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FMR1 knockout mice, enhanced signaling through mGluRs may disrupt this balance resulting in seizures. Consistent with this hypothesis, decreasing signaling through group I mGluRs (Yan et al., 2005; Dölen et al., 2007) or increasing signaling through GABA$_{\text{B}}$ receptors (this study) rescues audiogenic seizures in FMR1 knockout mice.

If audiogenic seizures result from an imbalance in mGluR and GABA$_{\text{B}}$-mediated signaling, we envisaged that seizures might be induced in wild-type mice by disrupting this balance pharmacologically. Although treating wild-type mice with the GABA$_{\text{B}}$ antagonist CGP 46381 did not induce seizures, the same dose of CGP 46381 elicited a high incidence of seizures in FMR1/RGS4 double-knockout mice. We hypothesize that, in wild-type mice, RGS4 regulates signaling through GABA$_{\text{B}}$ receptors. In FMR1/RGS4 double-knockout mice, mGluR signaling is enhanced because of the absence of FMRP. However, the loss of RGS4 regulation would be expected to increase inhibitory signaling through GABA$_{\text{B}}$ receptors, which would restore the auditory signaling balance and prevent seizures. This hypothesis presumes, at least in the context of seizures, a greater effect of RGS4 on GABA$_{\text{B}}$ signaling than mGluR-mediated signaling. Consistent with this idea, treating double-knockout mice with a GABA$_{\text{B}}$ antagonist reversed the protective effect and induced seizures; additionally, seizures could be induced in wild-type mice by treatment with an mGluR5-positive allosteric modulator together with a GABA$_{\text{B}}$ antagonist at doses that, when administered alone, did not elicit seizures. Together, these results provide evidence that audiogenic seizures result from an imbalance in mGluR and GABA$_{\text{B}}$ receptor signaling.

Given the role of RGS4 in regulating GPCR signaling, which is altered in fragile X, it is possible that genetic elimination of RGS4 may also reverse other fragile X phenotypes. RGS4 knockout mice show a relatively mild phenotype that includes sensorimotor deficits (Grillet et al., 2005) and cardiac abnormalities resulting from enhanced parasympathetic signaling (Cifelli et al., 2008). Expression of RGS4 mRNA has previously been reported to be decreased in the brains of FMR1 mice (Tervonen et al., 2005). Based on our findings, it is possible that this decrease is a compensatory response to increased mGluR signaling rather than a causative factor in the pathogenesis of fragile X. Although further study is needed to more precisely determine the role of RGS4 in fragile X, collectively, these observations indicate that RGS4 could be a potential target for treating fragile X syndrome.

Acknowledgments

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