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 mouse. 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 (Wiszewski 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

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.
GABA<sub>B</sub> Receptor-Mediated Signaling in Fragile X Syndrome

**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<sup>−/−</sup> 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 (Cifelli et al., 2008) was backcrossed seven generations onto the C57BL/6 background. FMR1 knockout mice on the C57/Bl6 background (Cifelli et al., 2008) was backcrossed seven generations onto the C57/Bl6 background (Cifelli et al., 2008) was backcrossed seven generations onto the C57/Bl6 background (Cifelli et al., 2008) was backcrossed seven generations onto the C57/Bl6 background. For the wild-type allele, primers S1 (5'-GTG TTG 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'-GGC GGC TCT ATG GCT TGT GGT GAT-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, OSET (5'SCA TCT TGA CCC AAA TCT GCC TGA G 3') and OSEF (5'GG CAT GAA ACA TGG GCT GGG GTT C 3') were used. For the knockout allele, OSET and OSEF (5'GGG CCA CCA 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 brain 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 CGC-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 Multilimage 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 x 17 x 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. 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-1H-pyrazol-5-yl) benzamide (CDPPB; Tocris...
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 differences.
wild-type mice were given an intraperitoneal injection of the
seizures in wild-type mice. At 27 to 30 days of age, male
results demonstrate that stimulating GABAB-mediated sig-
0.05) in seizure activity at both doses (data not shown). These
ment with 1.0 and 2.0 mg/kg (\(\text{mg/kg i.p.}\)) administered 45 min before seizure testing. Treat-
knockout mice showed statistically significant decreases (\(\text{p} < 0.05\)) and 79\% (\(\text{p} < 0.01\)) decrease, respectively, in
seizure activity (Fig. 3) compared with vehicle controls. When analyzed separately, both male and female FMR1
knockout mice showed statistically significant decreases (\(\text{p} < 0.05\)) in seizure activity at both doses (data not shown). These
results demonstrate that stimulating GABA\(_B\)-mediated sig-
aling rescues seizures in FMR1 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
reated with the GABA\(_B\) agonist (\(\text{R}\))-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 (\(\text{mg/kg}\))-baclofen produced a 67%
\(\text{p} < 0.05\) and 79\% (\(\text{p} < 0.01\)) degree, respectively, in
seizure activity (Fig. 3) compared with vehicle controls. When analyzed separately, both male and female FMR1
knockout mice showed statistically significant decreases (\(\text{p} < 0.05\)) in seizure activity at both doses (data not shown). These
results demonstrate that stimulating GABA\(_B\)-mediated sig-
aling rescues seizures in FMR1 knockout mice.

Treatment with a GABA\(_B\) Antagonist Induces Sei-
Zures 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
creasing GABA\(_B\) receptor-mediated signaling could in-
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). Al-
though 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 insuffi-
cient 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 (\(\text{p} < 0.05\)); this represented approxi-
mately a 5-fold increase in seizure incidence over vehicle
controls (Fig. 4). This finding suggests that genetically elim-
inating 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 Audi-
genic 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 in-
duce 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.
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 Gi/o-coupled signaling to inward rectifying potassium (KIR) 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 KIR 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 (Caspar 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 Ga_coupled receptors; activation of GABA_B receptors leads to reduced cAMP production and stimulates the opening of KIR channels, leading to hyperpolarization and increased membrane potential (Jacobson et al., 2007; Laboue`be 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-KIR signaling complexes in different cell types (Ja`en and Doupnik, 2006). RGS4 has been shown to interact with GABA_B receptors and KIR 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; Margareta-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...
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; Dolen et al., 2007) or increasing signaling through GABAB receptors (this study) rescues audiogenic seizures in FMR1 knockout mice. If audiogenic seizures result from an imbalance in mGluR and GABAB-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 GABAB 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 GABAB receptors. In FMR1/RGS4 double-knockout mice, mGluR signaling is enhanced because of the absence of the FRMR. However, the loss of RGS4 regulation would be expected to increase inhibitory signaling through GABAB 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 GABAB signaling than mGluR-mediated signaling. Consistent with this idea, treating double-knockout mice with a GABAB antagonist reversed the protective effect of increased seizures; additionally, seizures could be induced in wild-type mice by treatment with an mGluR5-positive allosteric modulator together with a GABAB 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 GABAB 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 somotoric deficiencies (Grillet et al., 2005) and cardiac abnormalities resulting from enhanced parasympathetic signaling (Cifelli et al., 2008). Expression of RGS4 mRNAs 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.

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References


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