Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP

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Abstract

Fragile X Syndrome is the most common form of inherited mental retardation worldwide. A Fragile X mouse model, $fmr1^{tm1Cgr}$, with a disruption in the X-linked *Fmr1* gene, has three substantial deficits observed in several strains: (1) sensitivity to audiogenic seizures (AGS), (2) tendency to spend significantly more time in the center of an open field, and (3) enlarged testes. Alterations in metabotropic glutamate receptor group I signaling were previously identified in the $fmr1^{tm1Cgr}$ mouse. In this study, we examined the effect of MPEP, an antagonist of the group I metabotropic glutamate receptor mGluR5, on audiogenic seizures and open field activity of $fmr1^{tm1Cgr}$ mice. Genetic analysis revealed synergistic reactions between $fmr1^{tm1Cgr}$ and inbred AGS alleles. In addition, AGS sensitivity due to the $fmr1^{tm1Cgr}$ allele was restricted during development. Examination of phenotypes combining mGluR5 inhibition and *Fmr1* mutation indicated that absence of FMRP may affect mGluR5 signaling through indirect as well as direct pathways. All strains of $fmr1^{tm1Cgr}$ mice tested (FVB/NJ, C57BL/6J, and an F1 hybrid of the two) had a more excitable AGS pathway than wild-type, and consequently required more MPEP to achieve seizure suppression. At high doses of mGluR5 antagonists, a Fragile X specific tolerance (loss of drug activity) was observed. The tolerance effect could be overcome by a further increase in drug dose. In open field tests, MPEP reduced $fmr1^{tm1Cgr}$ center field behavior to one indistinguishable from wild-type. Therefore, mGluR5 antagonists were able to rescue two of the major phenotypes of the FX mouse. Modulation of mGluR5 signaling may allow amelioration of symptoms of Fragile X Syndrome.
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1. Introduction

Fragile X Syndrome (FXS) is an X-linked disorder most often resulting from expansion of a CGG trinucleotide repeat found in the 5′-untranslated region of the *FMR1* gene. Once the expansion exceeds approximately 200 repeats, the *FMR1* gene becomes hypermethylated and transcriptionally inactive, a condition that results in the loss of FMRP protein production in the affected cell. The primary symptom of FXS is mental retardation. In addition, autistic and hyperaroused behaviors, including hyperactivity, hypersensitivity to sensory stimuli, and EEG abnormalities are observed. More than 20% of those with FXS develop childhood seizures (Musumeci et al., 1999). Attention deficits and anxiety in novel situations are also common.

A mouse model of Fragile X Syndrome, with a disrupted *Fmr1* allele designated $fmr1^{tm1Cgr}$ ("*fmr1*"), has been studied for the past decade (Bakker and Consortium, 1994). Biochemical analysis of the Fragile

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X (FX) mouse model has indicated that signaling through metabotropic glutamate receptors (mGluRs) may be altered. Changes in FMRP levels near synapses in response to mGluR group I signaling has been reported (Antar et al., 2004; Weiler et al., 1997). Of particular note for this study, it was recently reported that the CA1 region of $fmr1^{tm1Cgr}$ hippocampal slices exhibits increased long-term depression (LTD) when exposed to the mGluR group I receptor agonist DHPG (Huber et al., 2002).

LTD is an activity-dependent synaptic weakening which can be mediated at least in part by the group I mGluRs: mGluR1 and mGluR5. It had previously been shown using mGluR5 knockout mice that mGluR5 presence was required for mGluR1-mediated LTD (Huber et al., 2001); in contrast, LTD was reported to be normal in the CA1 region of mGluR1 knockout mice (Aiba et al., 1994). Furthermore, the mGluR5 agonist CHPG could induce LTD in the medial perforant pathway of the dentate gyrus (Camodeca et al., 1999), while the mGluR1 antagonist 4CPG alone did not block mGluR LTD (Oliet et al., 1997). Therefore, mGluR5 appeared to be essential for LTD in several assays.

Given the observation of altered LTD in $fmr1$ mice, FMRP might act downstream of mGluR group I receptors, in particular mGluR5, such that signaling is altered when FMRP levels are greatly reduced or lost. This raised the question, to be addressed in this study, whether in vivo evidence could be obtained for altered signaling through mGluR group I receptors in the $fmr1^{tm1Cgr}$ mouse.

MPEP (2-methyl-6-phenylethynyl pyridine hydrochloride) is a potent, specific, and blood brain barrier penetrable noncompetitive antagonist of mGluR5 receptors (Gasparini et al., 1999; Varney et al., 1999). MPEP suppresses clonic seizures induced by the mGluR5-selective agonist CHPG, and is also effective against sound-induced seizures in DBA/2 mice (Chapman et al., 2000). Therefore, MPEP was selected in this study for an initial assessment of the in vivo importance of mGluR5 receptor function in $fmr1^{tm1Cgr}$ mice. Two $fmr1$-specific and robust phenotypes were chosen for drug tests in FVB/NJ × C57BL/6 F1 hybrid mice: an elevated susceptibility to audiogenic seizures (AGS) and an increased tendency to move to the center of an open field (Yan et al., 2004).

It is shown here through the use of F1 hybrid mice that, in the absence of penetrant recessive modifier alleles, AGS sensitivity produced by the $fmr1^{tm1Cgr}$ allele is developmentally restricted, as is generally the case in human FXS. Furthermore, $fmr1^{tm1Cgr}$ mice have a more excitable audiogenic seizure pathway than wild-type littermates, and consequently, require more MPEP to achieve seizure suppression. Despite the complete acute effectiveness of MPEP in this AGS assay, $fmr1^{tm1Cgr}$ mice developed tolerance to MPEP's anticonvulsant

activity over a period of days. In the open field test, MPEP was found to have an effect opposite to its previously described anxiolytic effect, yet the drug caused $fmr1$ mouse center field behavior to become statistically indistinguishable from that of wild-type. Therefore, modulation of mGluR5 signaling may allow amelioration of symptoms of Fragile X Syndrome. Some of the findings reported here have been previously published in abstract form (Yan et al., 2003).

2. Methods

2.1. Animals

Male and female inbred FVB/NJ ("FVB") and C57Bl/6J ("C57") mice, and F1 hybrids ("hybrid") of the two strains, with or without an $fmr1^{tm1Cgr}$ allele ("$fmr1$" or "ko"), were used in the audiogenic seizure (AGS) testing. Data reported here are for the C57 male × FVB female cross; in several experiments the reverse cross was found to produce similar results. Male hybrids at one and three months of age were used for open field (OF) testing. The mice were housed in groups of five with a 12-h day–night cycle (lights on at 7:00 AM) with free access to mouse chow (PicoLab Mouse Diet 20) and water. Experiments were carried out between 11:00 AM and 2:00 PM (open field) and between 4:00 and 7:00 PM (AGS). Procedures involving animals and their care were reviewed by the Institutional Animal Care Usage Committee (IACUC) and were in accordance with ILAR guidelines.

2.2. Genotyping

Genotyping was performed as previously described (Yan et al., 2004). $fmr1^{tm1Cgr}$ mice were identified by neomycin phosphotransferase ("neo") signal, and wild-type were confirmed with exon 5 primers spanning the $fmr1^{tm1Cgr}$ disruption site.

2.3. MPEP

MPEP (2-methyl-6-phenylethynyl-pyridine hydrochloride; MW 229.71) was synthesized by Technically, Incorporated (Woburn, MA). It was stored as a powder at 4 °C and warmed up for 15–60 min before opening to avoid condensation. Resuspension was in distilled water to obtain a 5-mg/ml (21.7 mM) solution. A fresh stock solution was prepared for each experiment. Both MPEP and the distilled water vehicle were administered intraperitoneally (i.p.) into mice in a volume of 100–200 ml; 0.05 mg/kg to 30 mg/kg were used, a range believed to be specific in vivo (Pilc et al., 2002; Spooren et al., 2000b). (MPEP at 100 mg/kg in preliminary titrations gave the appearance of sedation and other

effects not apparent at lower doses.) MPEP was active in AGS assays from 15 min to 75 min after injection; tests reported here were conducted at 30 min.

2.4. Audiogenic seizure assay

Mice of 14–180 days of age (as specified in the text) were marked and exposed to a high intensity siren of frequency peak 1800–6300 Hz (Supplementary Fig. 1S) at an average sound pressure level of 125 dB at 11 cm (Personal Alarm, Model 49-417, Tandy Corporation) for up to 15 min in an empty, transparent plastic box ($28 \times 17.5 \times 12$ cm) with a sound absorbent tile lid under which the siren was mounted. The alarm was powered from a DC converter in order to ensure that sound pressure levels were maintained above 115 dB. The alarm frequency spectrum was obtained with Amadeus II software (HairerSoft) and an omnidirectional electret condenser microphone (ME-9, Olympus Optical) whose stated flat frequency response of 50–13,000 Hz was confirmed with white noise (Sound Studio). Seizures were scored by the time of occurrence (latency) and by type: clonic, tonic, tonic–clonic, status epilepticus ("SE"; a sustained tonic seizure most often resulting in respiratory arrest and death). AGS sensitivity refers to the population percentage exhibiting seizures. Audiogenic seizures generally began with a period of wild running and jumping. Wild running and jumping (WRJ) did not always lead to seizures; therefore, WRJ were not counted in seizure totals unless specifically noted. As clonic seizures were often subtle and subject to some variability in designation by observer, they were not analyzed in this study. Population status epilepticus percentage was used as a measure of AGS severity.

Median effective doses (ED_{50}) for AGS were estimated using ED50plus software (MH Vargas). In those cases (such as for male HYB wt primed mice) in which most doses did not produce responses (Fig. 3d), an estimated upper boundary ED_{50} was calculated by assuming that a single responding animal was observed in the non-responding group. χ^2 analysis supported the validity of the ED_{50} estimates. Non-responding MPEP groups were combined to compare vehicle and the total number of observations over a range of non-responding doses, with the understanding that ED_{50} must be less than the highest MPEP dose included in the non-responding group. For example, for the hybrid wt primed male mice, 3 of 21 vehicle treated mice seized, while none of 36 mice tested in the dose range 0.05– 1 mg/kg did (Fig. 3d). The difference between vehicle and MPEP treated groups had a Fisher's Exact Test $p = 0.04$. Since more seizing mice should have been observed at lower doses of MPEP, it can be conservatively estimated that the ED_{50} has a greater than 95% chance of being less than 1 mg/kg. The ED_{50} upper estimate obtained using ED50plus regression analysis

(described above) produced an ED_{50} estimate of <0.42 (Table 1).

2.5. Priming

On postnatal day 16 (C57 and hybrid strains) or 21 (FVB strains), the experimental group was placed in the AGS chamber and exposed to the siren for 15 s. Female mice were tested at the priming and peak ages established for males of the comparable strain. Testing occurred seven days after priming, which was found to be within the peak response window for all strains.

2.6. Open field testing

The open field arena was constructed from a 72×72 cm square box with 50 cm high walls as previously described (Yan et al., 2004), except that a floor line 4 cm from each wall was added to measure thigmotaxis. A clear acrylic sheet was used to cover the floor of the apparatus to facilitate cleaning between tests. The open field maze was placed on the floor of a room brightly lit with fluorescent lights. Male F1 hybrid (C57Bl/6J $\times$ FVB/NJ) mice were used as wild-type ("wt"). The same background carrying the $fmr1^{tm1Cgr}$ allele were also used and are designated "$fmr1$" (or "ko") in some text and figures. In the open field experiments, mice were aged 30–33 days ("1 month") or 90–95 days ("3 months"). The following numbers of mice were used in each treatment group: (a) 1 month $fmr1$: 20 vehicle, 11 MPEP 10 mg/kg, 10 MPEP 30 mg/kg, (b) 1 month wt: 20 vehicle, 10 MPEP 10 mg/ kg, 10 MPEP 30 mg/kg, (c) 3 month $fmr1$: 11 vehicle, 10 MPEP 30 mg/kg, and (d) 3 month wt: 10 vehicle, 10 MPEP 30 mg/kg. The animals were group-housed after weaning and genotyping. Mice were weighed and then MPEP administered intraperitoneally (i.p.). After injection, they were allowed to rest in their cages for 30 min prior to testing. Each mouse was then placed in a corner of the open field, and was videotaped for 5 min. Afterwards, the floor was cleaned with 70% ethanol. Videotapes were exported to a computer for analysis as previously described (Yan et al., 2004) except that films were scored by at least two observers. Experiments were blinded as follows: mice were given a coded identity

Table 1
Estimated MPEP AGS ED_{50}s

Strain	Non-primed male	Primed male	Non-primed female	Primed female
FVB*fmr*	12.0	17.5	8.8	8.0
FVB wt	i.s.	<8.5	n.s.	n.s.
HYB*fmr*	1.9	3.4	1.7	3.1
HYB wt	n.s.	<0.42	n.s.	n.s.

In mg/kg; n.s. indicates no seizures under the conditions employed; i.s. indicates insufficient seizures within dose ranges tested.

when brought to the testing room. A second person placed the mice into the open field, filmed them, and recorded the data. Locomotor activity was scored as total horizontal line crosses plus rearings (Yan et al., 2004).

2.7. Statistical analysis

Open field data were first analyzed using one-way ANOVA, since parametric statistical tests are more sensitive than non-parametric tests, and many parametric tests suffer only minimal inaccuracies when assumptions are not met; e.g. see Kiess, 2002. In those cases in which data were not normally distributed or for which variances among variables were not sufficiently similar (by F-test), and for which a transformation to resolve such issues was not found, the findings from parametric tests were confirmed with an appropriate non-parametric test (e.g. Kruskal–Wallis for one-way ANOVA). Based on post hoc analysis (Tukey–Kramer honestly significant difference test), the treatment most likely to account for a significant difference was dropped from the non-parametric distributions to confirm its role in producing the presumptive significant finding. For those experiments in which results were binomial (e.g. seizure or no seizure), data were analyzed using a binomial test, or Fisher's Exact Test (GB-STAT, Dynamic Microsystems) when conditions for standard χ^2 analysis were not met. In all experiments, the significance level was set at 0.05. In the figures, probabilities are denoted as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. "M" indicates mean, "SD" the standard deviation, and "η^2" provides an estimate of the strength of the effect, which is considered strong at or above 0.10–0.15 (Kiess, 2002). Results of most statistical tests are given in the Supplementary tables (indicated with a capital "S").

3. Results

3.1. fmr1^{tm1Cgr} AGS sensitivity is restricted during development

Male mice, 14–180 days old, were tested for sensitivity to audiogenic seizures (AGS) to determine the peak age of sensitivity. FVB *fmr1^{tm1Cgr}* mice were sensitive to AGS induction over a wide range of ages, from 14 days postnatally to 94 days; by 150 days of age, no further seizures were observed (Fig. 1). C57 *fmr1^{tm1Cgr}* mice also had a wide range of AGS sensitivity (from at least 15–47 days). By contrast, F1 hybrid (C57BL/6J × FVB/NJ) *fmr1^{tm1Cgr}* mice did not show seizures at 15 days or after 31 days (tested to 85 days; Fig. 1). Therefore, inbred *fmr1^{tm1Cgr}* mice had
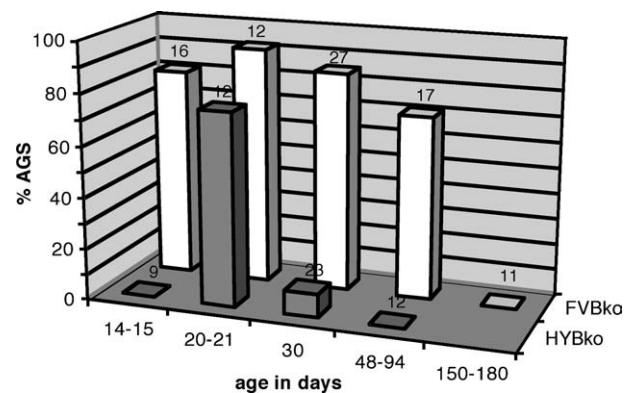

Fig. 1. Audiogenic seizure developmental course. Percent total audiogenic seizures (AGS) from 14 to 180 days of age. "FVBko" are FVB *fmr1^{tm1Cgr}* males and "HYBko" are F1 hybrid *fmr1^{tm1Cgr}* males. Numbers above bars indicate the total number of mice tested.

an extended period of AGS sensitivity throughout development.

AGS peak duration was also influenced by strain background. Males of the hybrid *fmr1^{tm1Cgr}*, C57 *fmr1^{tm1Cgr}*, and primed hybrid wild-type strains shared a seizure sensitivity peak at approximately 21 days of age, compared to 21–28 days for the FVB wild-type and FVB *fmr1^{tm1Cgr}* strains. Thus, FVB strains (wild-type and *fmr1^{tm1Cgr}*) showed a delayed peak of sensitivity well into the fourth week postnatally. Non-primed C57 and hybrid male wild-type strains did not show significant AGS under the conditions used here. Priming was required to provide sufficient hybrid wild-type mice with seizures for drug testing.

3.2. Inbred AGS alleles are detectable and synergistic with fmr1^{tm1Cgr}

Several findings were apparent from an examination of strains for total AGS and severity of seizures (Fig. 2). First, priming produced a clear trend to increase both total AGS and severity of seizures for all cases in which seizures were obtained (see Supplementary Information for statistical analysis and discussion). Second, the *fmr1^{tm1Cgr}* allele was much more potent in elevating seizure sensitivity and severity than priming.[1] Third, the FVB wild-type strain showed evidence of a strong seizure allele. A comparison of total AGS from primed FVB wild-type (14 AGS of 29 tested) and F1 hybrid wild-type (3 of 21) showed a significant difference by Fisher's Exact Test ($p = 0.012$). A simple explanation for the loss of AGS in the F1 hybrid, which has one

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[1] The following differences between primed (pr) and non-primed (npr) males were significant to $p < 0.001$ by Fisher's Exact Test: FVB primed vs. FVB *fmr1* non-primed for total AGS and status epilepticus, C57 primed vs. C57 *fmr1* non-primed for total AGS, and hybrid primed vs. hybrid *fmr1* non-primed for total AGS (Fig. 2).
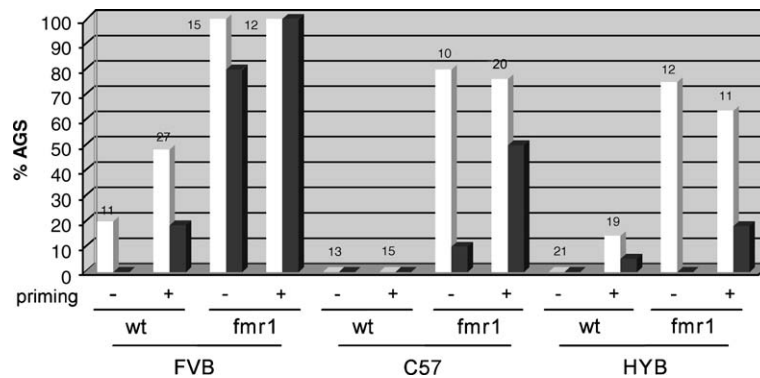
Fig. 2. AGS strain comparison for males. Lighter left bar in a pair shows total AGS as a percentage of the mice tested. Darker right bar in a pair shows the percentage mortality from status epilepticus, a measure of seizure severity. Priming was performed one week prior to AGS testing with a 15-s (non-seizure inducing) exposure to the AGS sound source. "fmr1" indicates $fmr1^{tm1Cgr}$ allele. FVB and C57 are inbred mouse strains FVB/NJ and C57Bl/6J. HYB is an F1 hybrid of the FVB and C57 strains. Numbers above bars indicate the total number of mice tested.

FVB parent, is that the FVB AGS allele is recessive. A comparison of the magnitude of FVB AGS allele effect in wild-type males with the $fmr1^{tm1Cgr}$ allele in hybrid males showed that they were statistically equal in producing status epilepticus and total AGS when primed, but that the $fmr1^{tm1Cgr}$ allele clearly was more potent in total AGS sensitivity in non-primed conditions: hybrid $fmr1^{tm1Cgr}$ (9 AGS of 12 tested) vs. FVB wild-type (2 of 10 tested) with the difference being significant with $p = 0.015$ by Fisher's Exact Test. There was no strong, direct evidence of a C57 AGS allele under the same conditions; only for the primed status epilepticus (SE) groups did C57 $fmr1^{tm1Cgr}$ trend towards greater AGS sensitivity than hybrid $fmr1^{tm1Cgr}$ mice ($p = 0.086$ by Fisher's Exact Test).

Both the FVB $fmr1^{tm1Cgr}$ and C57 $fmr1^{tm1Cgr}$ AGS status epilepticus responses were much larger than the sum of the inbred wild-type response plus the hybrid $fmr1^{tm1Cgr}$ response (Fig. 2). Therefore, a synergistic interaction between the inbred alleles and the $fmr1^{tm1Cgr}$ AGS allele was likely. For example, in comparing the levels of status epilepticus (the measure of AGS severity) between FVB $fmr1^{tm1Cgr}$ and hybrid $fmr1^{tm1Cgr}$ males, Fisher's Exact Test showed the increase in FVB $fmr1^{tm1Cgr}$ severity to be significant with $p = 0.0001$ (primed) or < 0.0001 (non-primed).

Female mice were qualitatively similar in AGS response to comparable males (Fig. 2S). The $fmr1^{tm1Cgr}$ allele enhanced AGS sensitivity substantially; however, the response to priming was much more variable by strain than for males.

3.3. $fmr1^{tm1Cgr}$ mice require more MPEP for AGS ED_{50} than wild-type mice

A comparison of wild-type and $fmr1$ dose—responses to MPEP is shown in Fig. 3. Such dose—effect titrations were used to estimate ED_{50}s (Table 1). Hybrid $fmr1^{tm1Cgr}$ male mice required much less MPEP for ED_{50} than FVB $fmr1^{tm1Cgr}$ males (5—10 mg/kg is below the 50% response level for the hybrids, while the same doses are above the 50% response level for FVB; Fig. 3; Table 1S). In three of four cases for $fmr1$ mice, ED_{50} was higher in primed compared to unprimed mice (Table 1), although the difference between primed and unprimed ED_{50} was small compared to that observed between wild-type and $fmr1$ ED_{50}s.

The ED_{50}s for HYB $fmr1^{tm1Cgr}$ mice, with and without priming, were comparable to and even slightly greater than those for FVB wt strains with and without priming (Table 1). This supports the findings presented above that the magnitude of the $fmr1^{tm1Cgr}$ allele on audiogenic seizure sensitivity is at least as great as that of recessive AGS allele(s) in the FVB strain. Female mice of hybrid and C57 $fmr1^{tm1Cgr}$ backgrounds, with or without priming, generally had lower ED_{50}s than males of comparable backgrounds (Table 1). A video, demonstrating the AGS behavioral phenotypes and the protective effect of MPEP is presented in the Supplementary Information.

3.4. Phenotypic difference between $fmr1^{tm1Cgr}$ and wild-type in open field exploratory behavior is extended

We had previously demonstrated that singly-housed male F1 hybrid mice of crosses between FVB/NJ and C57Bl/6J inbred strains showed a statistically significant difference in open field exploratory behavior from the same strains carrying the $fmr1^{tm1Cgr}$ allele (Yan et al., 2004). As this was one of the major phenotypic differences we found in such strains (along with elevated AGS sensitivity and macroorchidism), we wished to assess whether MPEP action on mGluR5 signaling would have
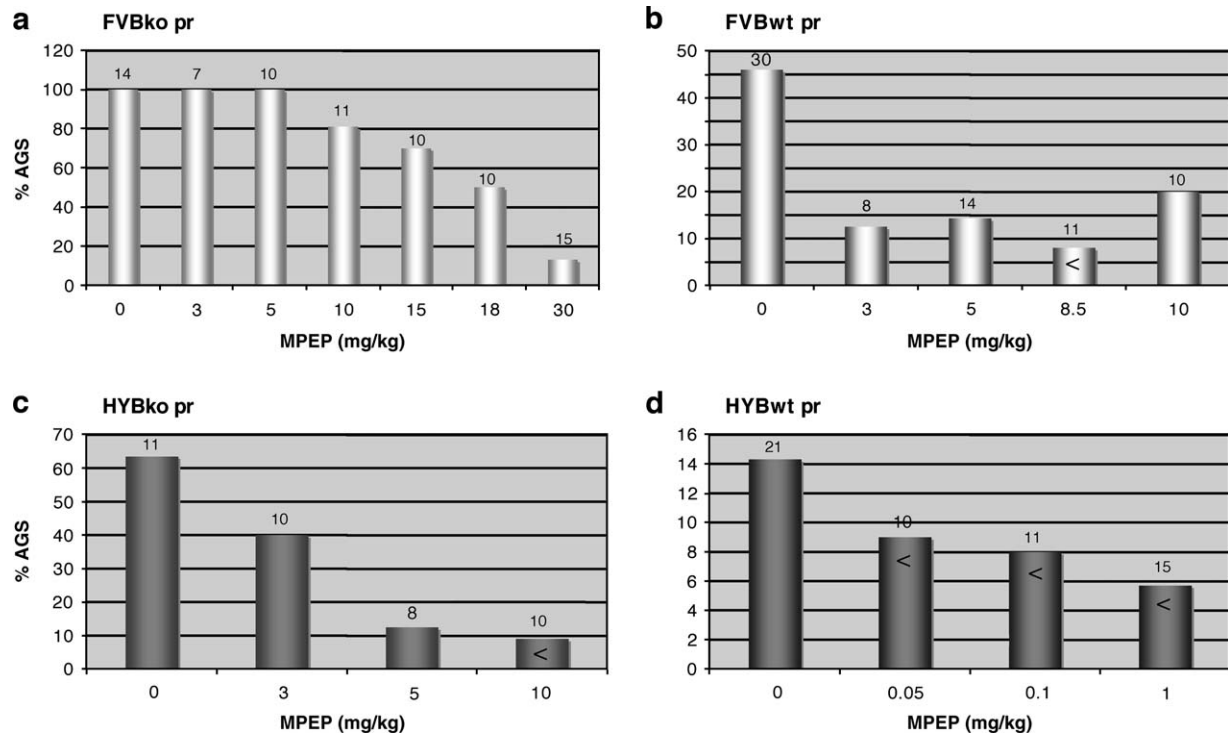
Fig. 3. MPEP dose titrations for AGS ED$_{50}$ estimation. Abbreviations: "ko" indicates *fmr1*[tm1Cgr] allele, "HYB" refers to F1 hybrid mice (C57BL/6J × FVB/NJ), "pr" indicates primed, and "% AGS" is the total audiogenic seizure percentage of the population. The number of mice tested is indicated over the bars.

a differential, and possibly corrective, impact on the genotypes, as shown above for AGS.

The difference between hybrid wild-type (wt) and *fmr1*[tm1Cgr] open field center square entry (CSE) and center square duration (CSD), previously shown in mice over 5 months of age (Yan et al., 2004), was also observed for mice injected i.p. with vehicle at both 1 month and 3 months. For 1-month-old vehicle-injected mice, a *t*-test of CSE measures showed a significant difference between *fmr1*[tm1Cgr] ($M = 6.1$, SD = 2.4) and wt ($M = 3.0$, SD = 2.3), ***$p < 0.001$, with a strong effect, $\eta^2 > 0.15$ (Table 2S). At 3 months, a similarly significant difference in CSE was also observed: *fmr1*[tm1Cgr] ($M = 6.1$, SD = 2.8) and wt ($M = 2.2$, SD = 1.6), **$p < 0.01$. As time in the center square (duration) was usually closely correlated to center square entry (i.e. the mice rarely stopped in the central square), it was not surprising to find that CSD measures at 1 and 3 months also showed significant differences by *t*-tests (Table 2S). Therefore, the open field exploratory behavioral differences previously reported for adult *fmr1*[tm1Cgr] and wt F1 hybrid mice (Yan et al., 2004) were confirmed and extended here to younger 1 and 3-month-old mice: *fmr1*[tm1Cgr] and wt vehicle-injected mice showed clear and statistically significant differences in the frequency with which they crossed into and stayed in the center of an open field. Complementary

differences were also quantified between the genotypes in thigmotaxis (wall-hugging; not shown).

3.5. MPEP causes fmr1[tm1Cgr] open field behavior to converge with that of wild-type

Having confirmed the phenotypic difference between *fmr1*[tm1Cgr] and wt mice at 1 month and 3 months, additional subjects were given MPEP to determine whether a differential response by genotype could be elicited. The significant differences between *fmr1*[tm1Cgr] and wt vehicle-injected CSE and CSD were eliminated with the administration of 10 mg/kg or 30 mg/kg MPEP (Figs. 4 and 5). ANOVA indicated that there was a significant difference among the treatments (Table 3S). Tukey–Kramer post hoc analysis was used to determine which pairs of treatment means were significantly distinct; the critical difference (CD) values are given in Tables 4–7S, along with asterisks indicating the significance level for the differences. The post hoc analysis showed that it was the *fmr1*[tm1Cgr] vehicle means at both ages, for both CSE and CSD, that were significantly different from wild-type vehicle and MPEP means in either strain. The analysis also indicated that MPEP did not significantly alter the behavior of the wild-type animals. Therefore, MPEP acted to rescue
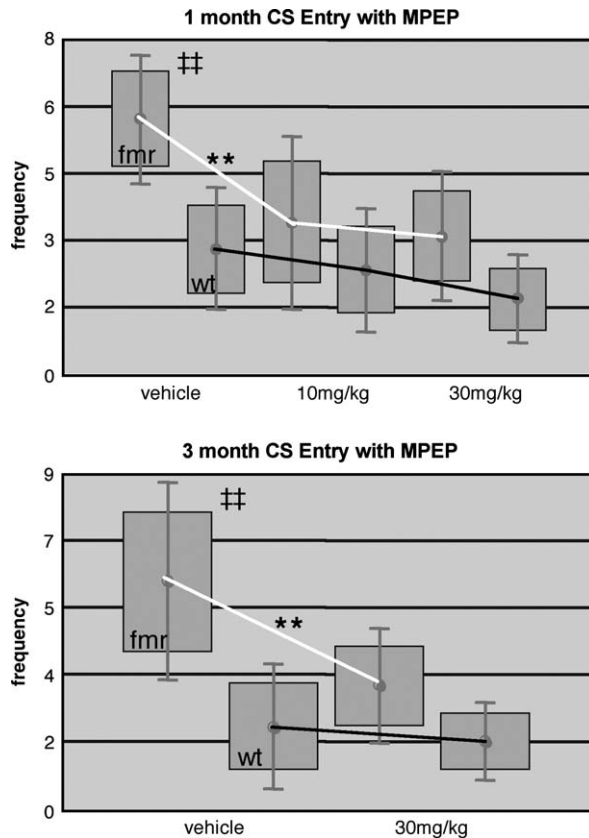
Fig. 4. Effect of MPEP on *fmr1*[tm1Cgr] center square entry. One-month-old and three-month-old male F1 hybrid *fmr1* and wt mouse entries into the center of an open field for 5 min. "##" between two treatment means, or "**" next to a line, indicates that the means of the two surrounding groups are from different distributions with *p* < 0.01. See Tables 3S–5S, and 8S for additional statistics.
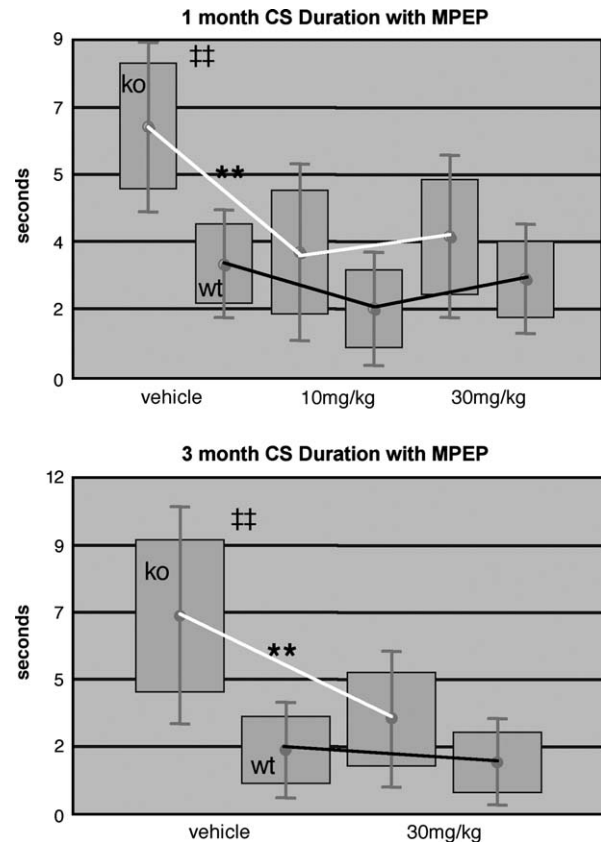

Fig. 5. Effect of MPEP on *fmr1*[tm1Cgr] center square duration. One-month-old and three-month-old male F1 hybrid *fmr1* and wt mouse time in the center of an open field during a 5 min period. "##" and "**" are as indicated in Fig. 4. See Tables 3S, 6S–8S for statistics.

the *fmr1*[tm1Cgr] open field phenotype and make it like wild-type.

These open field statistical results were confirmed using Kruskal–Wallis non-parametric analysis (see Section 2). For both CSE and CSD, at both 1 month and 3 months, there was a significant treatment effect, which was eliminated when the *fmr1*[tm1Cgr] vehicle group was dropped (Table 8S). The same MPEP effects on center square activity were observed in C57 strains (C. Sikorski and R.P. Bauchwitz, unpublished). Therefore, MPEP was causing the *fmr1*[tm1Cgr] phenotype to converge with that of wild-type.

MPEP was found to significantly diminish total locomotor activity in three of four groups tested: 1-month and 3-month-old *fmr1*[tm1Cgr] mice, and 3-month-old wild-type mice; 1-month-old wild-type mice did not show a significant decline. In the 1-month-old *fmr1*[tm1Cgr] mice, significantly reduced locomotor activity was already apparent at an MPEP dose of 10 mg/kg (means: vehicle = 214.4, 10 mg/kg = 172.9, and 30 mg/ kg = 160.1). Although we confirmed earlier findings that uninjected F1 hybrid wild-type and *fmr1*[tm1Cgr] mice

did not differ in total locomotor activity over the 5-min test period (Yan et al., 2004), when vehicle-injected, the wild-type mice had reduced activity at both 1 and 3 months of age.[2] Locomotor activity also significantly declined between 1 and 3 months of age for both wild-type and *fmr1* mice. When rearings were excluded to examine only horizontal mobility, the declines in locomotor activity with MPEP were only significant in the two *fmr1*[tm1Cgr] groups (1 month and 3 months of age), but not the two wild-type groups.

3.6. Repetitive MPEP exposure induces tolerance in *fmr1*[tm1Cgr] mice

It was of interest to determine how repeated exposure to MPEP would affect activity of the compound against AGS. MPEP was applied by i.p. injection under the following conditions: (1) as a single dose, (2) on the 5th day after four days of single

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[2] One-month uninjected wt *M* = 211.5, SD = 44.9, *fmr1* *M* = 230.2, SD = 48.6, *t*(40.3) = 1.28, *p* = 0.73; 1-month vehicle-injected wt *M* = 168.5, SD = 36.9, *fmr1* *M* = 214.4, SD = 46.5, *t*(37.9) = −3.37, *p* = 0.002.

vehicle injections, or (3) as single daily i.p. injections over 5 days (Fig. 6). The AGS assay was performed after the last dose. Total AGS, death from status epilepticus ("SE"), and latency to first seizure (or wild

running and jumping in the absence of clonic or tonic seizures) were recorded.

There was no significant difference in giving FVB $fmr1^{tm1Cgr}$ mice a single dose of MPEP and giving the same as a single dose after four days of water injections (Fig. 6a). However, MPEP given for 5 days gave significantly less protection than MPEP given after four days of water injections (by χ^2 Test for Independence, or Fisher's Exact Test when necessary – see Section 2). The latency to seizure (not shown) was also significantly reduced after 5 days of MPEP compared to a single dose (Tukey–Kramer **CD = 31.36, and Kruskal–Wallis **$p = 0.004$).

In contrast to the findings for FVB $fmr1^{tm1Cgr}$ mice, primed wild-type FVB male mice given 5 days of i.p. MPEP did not show tolerance to the effects of MPEP compared to uninjected, primed FVB male mice (Fig. 6a).

In order to assess whether the tolerance effect was common to other mGluR5 antagonists, a proprietary mGluR5 specific antagonist ("Compound A") was given at 30 mg/kg to FVB wt and $fmr1^{tm1Cgr}$ mice (Fig. 6b). Compound A produced an even larger tolerance effect in the FX mice than did MPEP, despite its having a much longer half-life (at least 10 h in other behavioral tests, compared to an MPEP half-life in the AGS assay of approximately 45 min). As for MPEP, Compound A showed no tolerance in FVB wt mice.

MPEP was also given to HYB $fmr1^{tm1Cgr}$ mice at 20 mg/kg and 30 mg/kg in order to determine whether there were strain or dosage effects (Fig. 6c). MPEP showed a strong tolerance response at 30 mg/kg MPEP, but showed none that was detectable at 20 mg/kg.

Finally, in order to determine if high dose, FX specific tolerance could be overcome with a subsequent increase in drug dose, MPEP was given for 4 days at 30 mg/kg, then on the 5th day at 60 mg/kg prior to AGS testing. The results showed full protection (no tolerance; $p = 0.016$ in a binomial test).

4. Discussion

Several prior studies have suggested a link between mGluR group I signaling and FMRP: (1) FMRP levels were increased in wild-type synaptosomes and cultured cortical neurons after stimulation by the mGluR grI agonist DHPG (Todd et al., 2003a; Weiler et al., 1997), (2) MPEP substantially blocked a KCl-stimulated movement of FMRP and $Fmr1$ mRNA into dendrites of cultured hippocampal cells, while stimulation by DHPG led to a decrease in FMRP at synapses (Antar et al., 2004), and (3) increases in barrel-cortex FMRP levels from whisker stimulation were blocked by NMDAR and mGluR group I antagonists (MK-802 and AIDA, respectively; Todd et al., 2003b). Other
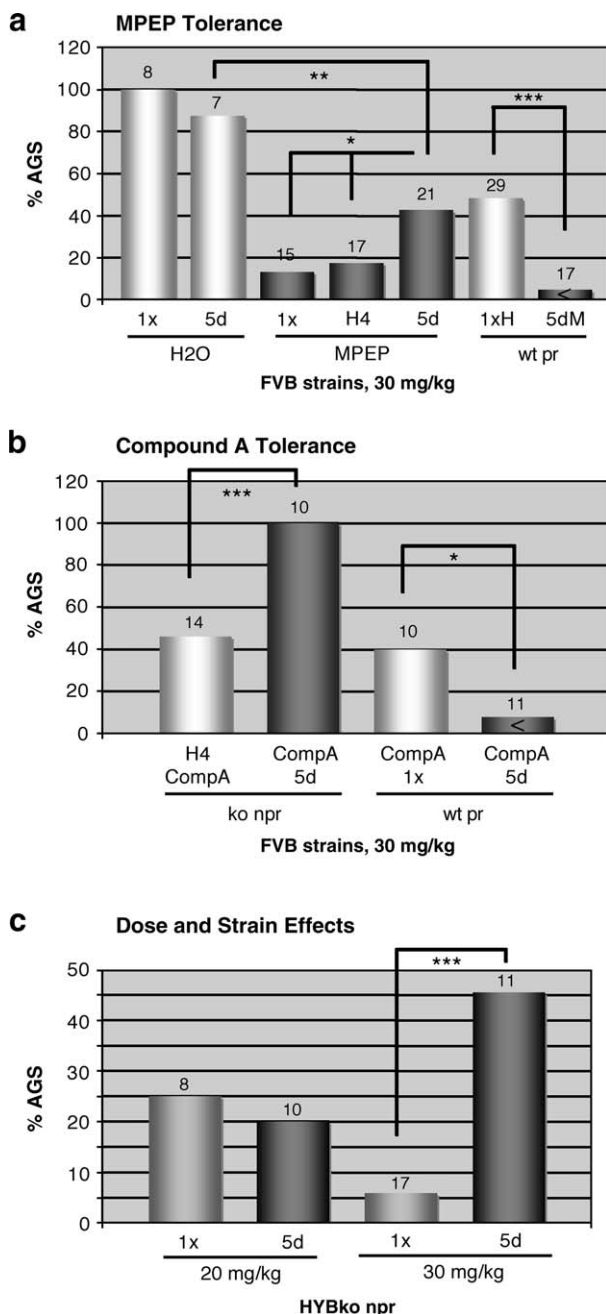
Fig. 6. mGluR5 antagonist tolerance tests. (a) Percentage of exposed population of FVB $fmr1^{tm1Cgr}$ ("ko") and wild-type primed ("wt pr") males showing audiogenic seizures (AGS) after a single injection of ("1x"), or multi-day exposure ("5d") to MPEP ("M") or vehicle (H₂O). "H4" indicates that four daily injections of vehicle were given prior to an injection of MPEP on the 5th day. (b) Fragile X specific tolerance to a proprietary mGluR5 antagonist, "Compound A" ("CompA"). (c) High dose MPEP tolerance in non-primed F1 hybrid $fmr1^{tm1Cgr}$ males ("HYBko npr"). Statistical comparisons were made using χ^2 Test of Independence, or as necessary, Fisher's Exact Test. Numbers above bars indicate the total number of mice tested.

experiments have found signs of altered mGluR group I signaling in FMRP-deficient mice: (1) DHPG-induced LTD was elevated in hippocampal slices from $fmr1^{tm1Cgr}$ mice (Huber et al., 2002), while (2) DHPG-stimulated PSD-95 translation was lost in $fmr1^{tm1Cgr}$ cultured cortical neurons (Todd et al., 2003a). These findings linking FMRP and mGluRs at the synapse build upon earlier evidence that both FMRP and mGluRs have important roles in synaptic plasticity. For example, absence of FMRP appears to negatively affect synaptic morphology in both humans and mice (Comery et al., 1997; Hinton et al., 1991; Irwin et al., 2001; Jenkins et al., 1984; Nimchinsky et al., 2001; Rudelli et al., 1985), while metabotropic glutamate receptors have a well known role in long-term synaptic plasticity, including induction of LTP in the hippocampus, and LTD at the parallel-Purkinje cell synapse of the cerebellum (Anwyl, 1999; Bashir et al., 1993; Bortolotto et al., 1994; Bortolotto and Collingridge, 1992; Linden, 1994; McGuinness et al., 1991; Zheng and Gallagher, 1992). Interestingly, DHPG stimulation has been demonstrated to produce elongated spines in cultured hippocampal neurons (Vanderklish and Edelman, 2002).

Therefore, we have been interested in assessing the effects of compounds which could modulate mGluR group I activity on cognitive and behavioral phenotypes in Fragile X systems in vivo. In a previous study, we performed a phenotypic survey of $fmr1^{tm1Cgr}$ mice on an F1 hybrid genetic background (FVB/NJ × C57BL/6J) in order to reduce or eliminate the recessive effects from inbred genetic loci (Yan et al., 2004). It was observed that the hybrid $fmr1^{tm1Cgr}$ mice had strongly elevated AGS susceptibility, and substantial behavioral differences from wild-type in the open field and Barnes maze. As the AGS and open field differences had also been seen in inbred $fmr1^{tm1Cgr}$ strains (Chen and Toth, 2001; Musumeci et al., 2000; Peier et al., 2000), it was these tests which were used here to assess the behavioral effects of various pharmacologic interventions.

4.1. $fmr1^{tm1Cgr}$ F1 hybrid mice become seizure resistant prior to adulthood

FVB $fmr1^{tm1Cgr}$ mice were the most AGS sensitive strain as measured by population frequency and severity of seizures. Furthermore, they had an extended period of AGS susceptibility. In contrast, the hybrid $fmr1^{tm1Cgr}$ mice lost AGS sensitivity before adulthood (after four weeks of age). Becoming seizure resistant with maturity is consistent with the clinical experience that most FXS patients grow out of their seizures by the end of adolescence (Wisniewski et al., 1991). Therefore, we conclude that the enhanced and extended period of AGS sensitivity in the inbred FVB strain background reflects the actions of a recessive locus acting together with the $fmr1^{tm1Cgr}$ allele. This suggests that the true Fragile X

AGS phenotype is revealed more accurately on the hybrid background. In addition, hybrid $fmr1^{tm1Cgr}$ mice showed audiogenic seizure susceptibility at least equivalent to that of the FVB wild-type inbred strain, demonstrating that the $fmr1^{tm1Cgr}$ mutation is a bona fide AGS susceptibility locus like that found in the FVB strain, and not simply a modifier of other recessive AGS loci of inbred mice. Hybrid wild-type animals were essentially resistant to AGS under our conditions unless primed, a phenomenon also consistent with the action of recessive modifier loci in the inbred lines.

It is known that susceptibility to seizure activity is highly dependent on the developmental state of the brain, particularly the maturation of inhibitory circuits occurring during the second week of mouse postnatal development (e.g. see Michelson and Lothman, 1989). Consequently, the substantial difference in age of priming and AGS peaks observed among the strains may reflect a difference in the timing of brain development. Similarly, it is well known that male and female adolescent mammals do not develop at the same rate. Therefore, differences between males and females of a strain in AGS sensitivity might reflect differences in timing of brain development. More direct strain or estrous cycle (catamenial) effects of neurosteroids on AGS suppression are also possible (Reddy, 2004).

The developmental responses observed in this study can also be compared to those in two earlier studies examining AGS in $fmr1^{tm1Cgr}$ mice. The first study of AGS in non-primed $fmr1$ mice was performed on the FVB background from 17 to 45 days of age (Musumeci et al., 2000). It was observed that $fmr1$ mice retained high seizure sensitivity into adulthood, as also reported here. In a subsequent study, it was reported that no convulsions were obtained in FVB $fmr1^{tm1Cgr}$ mice before the age of 10 weeks (70 days); AGS sensitivity then increased to 70% in 20–34-week-old animals (168–238 days) (Chen and Toth, 2001). This is a time course dramatically shifted into the adult period from what was reported here, perhaps the result of differences in the auditory stimulus or unanticipated sound conditioning. The data presented in this study suggest that it would be prudent to restrict FX AGS tests to the first month of life, as non-$fmr1^{tm1Cgr}$ effects may predominate at later times in the FVB strains.

4.2. Strain AGS sensitivity correlates with MPEP ED_{50}

The ED_{50} estimates suggested several trends: (1) that $fmr1$ mice always had a much higher ED_{50} than wt, (2) that hybrid mice had much lower ED_{50}s than inbred FVB strains, (3) that primed mice most often had a higher ED_{50} than unprimed, and (4) that females had a tendency towards lower ED_{50} than comparable males.

Furthermore, the more sensitive a strain was to AGS, the greater the amount of MPEP required to reduce the seizure frequency by 50% (ED_{50}). The simplest explanation for these results in the *fmr1* mice is that there is an increase in mGluR-dependent signaling in the *fmr1*[tm1Cgr] auditory system. Since FMRP has been demonstrated to act as a translational repressor of its synaptic cargo mRNAs (Laggerbauer et al., 2001; Li et al., 2001; Zalfa et al., 2003), a loss of FMRP could lead to an increase in translation of an unknown protein which enhances signaling in mGluR5 pathways. Support for this view comes from a study of *fmr1*[tm1Cgr] hippocampal neurons in F1 hybrid hippocampal slices; the *fmr1*[tm1Cgr] neurons showed prolonged ictal discharges which were returned to a wild-type phenotype in the presence of MPEP (Chuang et al., 2004). A variety of other suggestive linkages have been noted and proposed as an "mGluR theory of Fragile X mental retardation" (Bear et al., 2004).

4.3. MPEP corrects fmr1 open field behavior to wild-type

It is known that alterations of mGluR signaling can affect motor activity in mice. The striatum has one of the highest concentrations of mGluRs in the CNS (Attarian and Amalric, 1997). The nucleus accumbens, located in the ventral striatum, is important for motor activity and receives glutamatergic inputs from the prefrontal cortex, hippocampus, and amygdala (Meeker et al., 1998). DHPG-induced rats to rotate when infused into the striatum, while group I mGluR antagonists diminished contralateral rotation (Kearney et al., 1997). Therefore, it may be that the tendency for FX mice to enter the open field more than wild-type is related to an enhanced group I mGluR activity in the striatum, coupled with unilateral stimulation, such as might occur from whisker contact with walls during thigmotaxis.

The ability of MPEP to reverse the tendency of C57 and F1 hybrid *fmr1*[tm1Cgr] mice to make substantially more crossings of, and spend more time in, the center of an open field (Peier et al., 2000; Yan et al., 2004) was examined. In experiments presented here, MPEP significantly reduced *fmr1* but not wild-type center square entries and duration. Of particular note, the effect of MPEP on *fmr1* animals was of sufficient magnitude that *fmr1* entries and duration converged with those of wild-type animals so that the difference became statistically insignificant.

MPEP has been used in rodent open field tests previously to assess its effect on locomotor activity. However, prior tests have varied so significantly in terms of time in and form of the field, MPEP doses, species, strains, and ages, that at least six reports are evenly divided in finding that MPEP induced increases, decreases, or no change in locomotor activity (McGeehan et al., 2004; Nadlewska et al., 2002; Ossowska et al., 2001; Petersen et al., 2002; Spooren et al., 2000a,b). In this study, MPEP produced a significant reduction of total locomotor activity in three of four groups tested, at both 10 mg/kg and 30 mg/kg. Declines in horizontal line crossing were only significant for the *fmr1*[tm1Cgr] mice.

No prior reports of MPEP's effects on center square behavior have been made, to our knowledge. One possible explanation for MPEP's action in the open field tests is that it generally diminished locomotion. Even so, it appears that, as for the AGS tests, the *fmr1*[tm1Cgr] mice had a quantitatively different response to the drug, consistent with underlying differences in mGluR5 signaling. Currently the most common interpretation of elevated center square activity (or a decrease in thigmotaxis), is that it reflects a reduced level of anxiety (Prut and Belzung, 2003; Simon et al., 1994). It is unlikely that the change in phenotype reported here is related to MPEP's observed anxiolytic effects (Spooren et al., 2000b), since reduced anxiety would have been expected to increase time in the open field, the opposite of what was observed. Humans with FXS show substantially elevated indices of anxiety, particularly in novel situations; yet if the open field behavior of the *fmr1*[tm1Cgr] mice is interpreted to reflect anxiety, then they would appear to be less anxious than their wild-type littermates, as previously noted (Peier et al., 2000). However, use of drugs known to be anxiolytic in humans in the open field assay with rodents frequently produces no response, or even an anxiogenic response (Prut and Belzung, 2003), so the interpretation of open field test behavior can be uncertain. In contrast, the elevated plus maze (EPM) is considered a strong assay for anxiety in the rodent since drug responses apparently correlate well with those in humans (Pellow and File, 1986). In a prior study, we did not find any difference between wild-type and *fmr1*[tm1Cgr] mice in the EPM (Yan et al., 2004). Multiple factors affecting open field activity, including separate exploratory and anxiety drives, have been previously proposed (Ramos et al., 1997; Russell, 1973; Walsh and Cummins, 1976). Therefore, we suggest that any difference in anxiety between wild-type and *fmr1*[tm1Cgr] mice may be overshadowed by the differences in exploratory tendencies or other traits.

4.4. Tolerance to MPEP can develop

Repeated administration of MPEP led to diminished protection against AGS. This contrasts with the lack of tolerance observed with repeated administration of MPEP in anxiety and depression assays (Pilc et al., 2002). However, tolerance in anxiety assays has been shown in a related compound, MTEP (Busse et al., 2004). There are many mechanisms by which tolerance to pharmacologic agents can arise, including alterations in drug clearance, receptor number, or distal response elements. With respect to alterations in mGluR5 levels,

preliminary immunohistochemical and Western blot examinations of the inferior colliculus after acute administration of 0 or 30 mg/kg MPEP suggested that mGluR5 levels are being rapidly altered in response to MPEP administration and sound exposure (Q.J. Yan and R.P. Bauchwitz, unpublished). Therefore, it might be that repeated and sufficiently depressed mGluR5 signaling by high doses of antagonists, could produce elevations of active mGluR5. Increased mGluR5 levels would be consistent with the observation made here that increasing the ultimate MPEP dose prior to testing could overcome the tolerance effect. That our data also indicate that the tolerance is essentially FX specific suggests that mGluR5 levels could be regulated in part by FMRP.

4.5. MPEP's point of intervention

A direct, cell autonomous relationship between MPEP's effect on mGluR5 and the in vivo rescue of the $fmr1^{tm1Cgr}$ AGS and open field phenotypes would appear to be a straightforward proposition. Both mGluR5's effects on LTP and LTD, and FMRP's action as a translational inhibitor, are linked to synaptic protein synthesis. Fig. 7 summarizes some of the published findings relevant to the idea that mGluR5 acts upstream of, and through action on, FMRP. DHPG stimulation of normal mouse neurons in culture has led to an increase in PSD-95 translation (Todd et al., 2003a) and FMRP and Fmr1 trafficking (Antar et al., 2004). When a single disruption to the pathway was introduced, the phenotypes were altered, i.e. MPEP prevented activity-dependent (stimulated) FMRP movement, and $fmr1^{tm1Cgr}$ prevented an activity-dependent increase in PSD-95 levels. Thus, the data are consistent with proposals that mGluR5 stimulation by sensation (e.g. sound) or agonists (e.g. DHPG) would produce a signal which caused FMRP to release its cargo mRNAs for translation near the activated synapse (Antar et al., 2004; Todd et al., 2003a). In the absence of FMRP, no mGluR group I signals would be transmitted and negative phenotypes would result.

None of the experiments published prior to this study, however, have examined MPEP's activity in the context of the $fmr1^{tm1Cgr}$ allele. In effect, MPEP, as an antagonist of mGluR5, acts to produce a negative mutation of the receptor's activity. In combination with $fmr1^{tm1Cgr}$, also considered a negative mutation (Yan et al., 2004), it is possible to get some sense of what a double mutant's phenotype would be in vivo. In this study, when MPEP was applied to $fmr1^{tm1Cgr}$ mice, it was able to restore seemingly normal behavior. However, the $fmr1^{tm1Cgr}$ mutation should effectively break any normal connection between mGluR5 and the downstream phenotype if it is required to transmit mGluR5's signals (Fig. 7). Consequently, it is unlikely that any
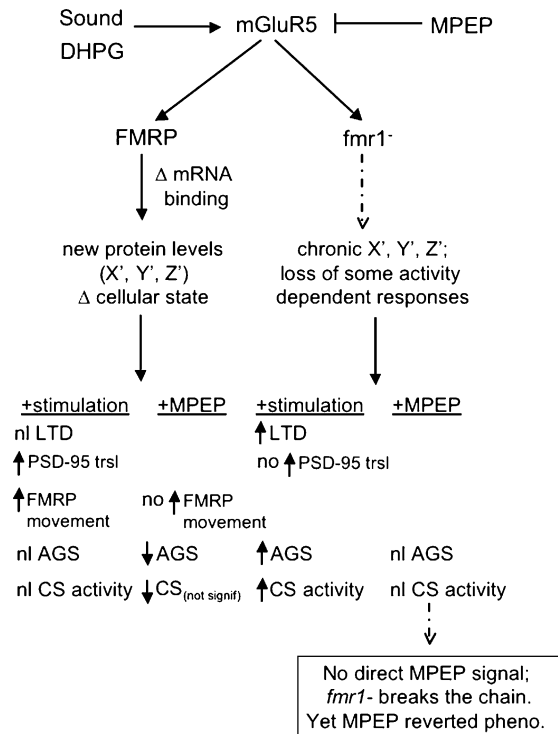

Fig. 7. MPEP and $fmr1^{tm1Cgr}$ effect summary. MPEP blocks the actions of sensory stimuli (e.g. sound) or agonists (e.g. DHPG) on mGluR5. One-way arrows from mGluR5 to FMRP or $fmr1^-$ indicate the possibility that mGluR5 stimulation and blockage produce downstream effects by influencing FMRP. Upon receiving synaptic signals, such as from mGluR5, FMRP is thought to release mRNA cargos it carries to allow their translation. In the absence of FMRP ($fmr1^-$), some synaptic protein levels are altered and some loss of synaptic activity-dependent responses are observed, e.g. no DHPG stimulation dependent increase of PSD-95 (Todd et al., 2003a). Other pathologic effects of $fmr1^-$ include increased LTD in the hippocampal CA1 region (Huber et al., 2002). In this study, it is demonstrated that MPEP can reverse pathologic effects of FMRP absence ($fmr1^-$); however, MPEP is unlikely to act by blocking mGluR5 signaling to an FMRP not present to receive such signals ($fmr1^-$). Fig. 8 illustrates alternate signaling pathways. MPEP effect on FMRP movement: Antar et al. (2004). AGS: audiogenic seizures. CS: center square activity.

altered mGluR5 signal caused by MPEP would be able to act through an FMRP no longer present to restore a normal phenotype. Therefore, MPEP may not act solely upstream of, or directly dependent upon, FMRP. This interpretation is consistent with those of an earlier study in which, rather than eliminating a phenotype (LTD) from tissue slices, the $fmr1$ mutation led to an increase in signaling (Huber et al., 2002). That finding led to the proposal that mGluR group I signaling could act through non-FMRP dependent products of protein translation, and that FMRP's presence was required to provide negative feedback on mGluR group I signaling. Fig. 8 illustrates this possibility, and emphasizes that an alternate path for mGluR5 signaling must exist.

It is also conceivable that MPEP could produce some actions indirectly. For example, MPEP might reduce

glutamatergic signaling in the auditory pathway, in general, regardless of the actual deficit producing the AGS sensitivity (see Supplementary Information for additional discussion). If Fragile X Syndrome led to decreases in inhibitory neural systems, then elevated seizure susceptibility would be expected, as would the MPEP effects on AGS observed here (Fig. 8). Furthermore, as inhibitory systems in the brain mature in mice during the second and third weeks of postnatal life (Michelson and Lothman, 1989), $fmr1^{tm1Cgr}$ AGS sensitivity should diminish, as was observed in this report. In this light, reduced inhibition would be a natural response to an underlying problem in FXS, i.e. reduced or inadequate neuronal responsiveness. Thus, a developmental hyporesponsive explanation for the core Fragile X deficit would still be predicted to produce many of the seizure and other secondary symptoms of FXS. Damping down neuronal network responses would be expected to prevent AGS and possibly even anxiety, hyperarousal, and related symptoms.

Fig. 8 illustrates the potentially indirect interaction between $fmr1^{tm1Cgr}$ and mGluR5 in a disinhibited neuronal environment. Loss of FMRP during develop-
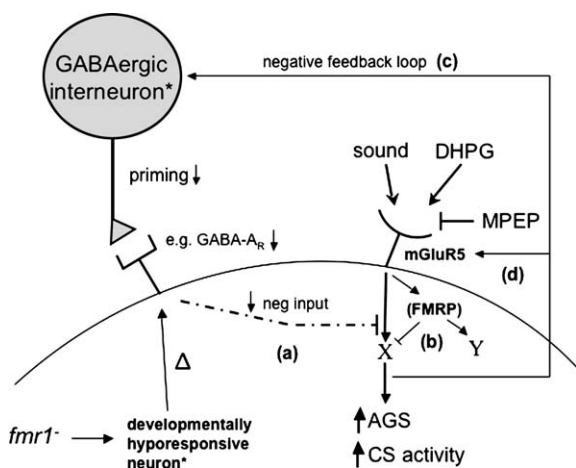
Fig. 8. Alternate pathways for MPEP action. The $fmr1^{tm1Cgr}$ allele may act indirectly, as well as directly, to modulate mGluR5 signaling. In the example proposed here based on the effects of AGS priming in reducing inhibitory tone in the inferior colliculus, absence of FMRP leads to a developmentally hyporesponsive state in some neurons, including both GABAergic interneurons or excitatory ones. A compensatory disinhibition may result, with decreased negative signaling into the mGluR5 pathway (a). The consequent imbalance from loss of negative feedback, possibly at the level of neuronal circuitry (c), leads to a pathological state (increased AGS and CS activity). Direct loss of negative feedback regulation on the mGluR5 pathway in the absence of FMRP is also illustrated (b), as previously proposed (Huber et al., 2002). FMRP-independent signaling from mGluR5 is shown (through X), as well as the ability of mGluR5 to act through FMRP directly to cause downstream effects (Y). MPEP can dampen excess signaling through X, but may not reverse effects on Y (see Fig. 7). High dose Fragile X specific mGluR5 antagonist induced tolerance potentially acting through pathway X is indicated in (d). AGS: audiogenic seizures. CS: center square activity.

ment could lead to a reduction in negative neuronal inputs (Fig. 8a or c). If basal neuronal signaling in FXS is not elevated, a loss of negative feedback would be the more likely proposition. In each case, FMRP would, in effect, be working upstream of mGluR5 to produce elevated signaling from it. A reduction of negative feedback circuit action would make the mechanism of $fmr1^{tm1Cgr}$ action very similar to that proposed for audiogenic priming and inbred AGS alleles (see Supplementary Information). This does not rule out the previously proposed direct negative feedback of FMRP on mGluR5 signaling (Fig. 8b; Huber et al., 2002); both direct and indirect FMRP effects may exist. Of note, however, our data indicate that mGluR5 must be able to signal, even if pathologically, in the absence of FMRP. This is indicated by the "X" pathway in Fig. 8b. As data exist suggesting that some neuronal responses may involve mGluR5 signaling acting primarily through FMRP (Antar et al., 2004; Todd et al., 2003a), a "Y" pathway is shown as well (Fig. 8). For phenotypes, such as those studied in this work, which can be influenced through a relatively FMRP-independent "X" pathway, or which result from indirect effects of disinhibition, MPEP should be able to prevent the expression of aberrant changes induced by loss of function mutations of *Fmr1*. However, it might also be predicted that not all phenotypes in which mGluR5 acts through FMRP, e.g. via the "Y" pathway, will be corrected by MPEP in *fmr1* cells. The Fragile X specific tolerance described here may be another in vivo example of action through pathway X, as it could be produced by the absence of FMRP's action as a translational brake allowing an elevation in active mGluR5 (Fig. 8d).

In summary, we report that: (1) mGluR5 antagonists can reverse major phenotypes of an FX mouse model, (2) the FX mouse phenotypes are produced through a pathway not directly signaling through FMRP, and (3) high doses of mGluR5 antagonists can produce a Fragile X specific tolerance effect. Thus, mGluR5 antagonists are able to replace FMRP as an mGluR5 signaling damper to revert the FX phenotype to wild-type. Compounds modulating mGluR activity in vivo should, at a minimum, have beneficial effects for treating the hyperarousal symptoms, including seizures and anxiety, of Fragile X Syndrome. Related symptoms seen in other disorders, such as autism, might also benefit from use of such drugs. Finally, we anticipate that additional experiments in Fragile X animal models should allow determination of whether mGluR-modulating agents might be of even more fundamental benefit in the treatment of Fragile X Syndrome, including its cognitive deficits.[3]

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[3] See Supplementary Information on the NeuroPharmacology web site for discussion.

Acknowledgements

Appendix A. Supplementary information

Supplementary information for this manuscript can be downloaded at doi: 10.1016/j.neuropharm.2005.06.004.

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