The GABA_A Receptor Agonist THIP Ameliorates Specific Behavioral Deficits in the Mouse Model of Fragile X Syndrome

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Key Words

Fragile X syndrome • GABA_A receptor agonist • Behavior • Developmental disabilities • Mouse model

Abstract

Hyperactivity, hypersensitivity to auditory stimuli, and exaggerated fear are common behavioral abnormalities observed in individuals with fragile X syndrome (FXS), a neurodevelopmental disorder that is the most common genetic cause of autism. Evidence from studies of the *Fmr1* knockout (KO) mouse model of FXS supports the notion that impaired GABAergic transmission in different brain regions such as the amygdala, striatum or cerebral cortex is central to FXS behavioral abnormalities. This suggests that the GABAergic system might be an intriguing target to ameliorate some of the phenotypes in FXS. Our recent work revealed that THIP (gaboxadol), a GABA_A receptor agonist, can restore principal neuron excitability deficits in the *Fmr1* KO amygdala, suggesting that THIP may also restore some of the key behavioral abnormalities in *Fmr1* KO mice. Here, we reveal that THIP significantly attenuated hyperactivity in *Fmr1* KO mice, and reduced prepulse inhibition in a volume-dependent manner. In contrast, THIP did not reverse the deficits in cued fear or startle response. Thus, this study shows that enhancing GABAergic transmission can correct specific behavioral phenotypes of the *Fmr1* KO mouse further supporting that tar-

geting the GABAergic system, and specifically tonic inhibition, might be important for correcting or ameliorating some key behaviors in FXS.

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Introduction

Fragile X syndrome (FXS) is a rare genetic disorder that affects 1:4,000 males and 1:8,000 females [1]. Those with FXS display intellectual disabilities and exhibit a wide repertoire of behavioral abnormalities such as hyperactivity, abnormal social interactions, exaggerated fear, anxiety, and hypersensitivity to auditory stimuli. Collectively, this makes the fragile X mental retardation 1 (*FMRI*) the most prominent single gene associated with autism [2]. FXS is caused by the transcriptional inactivation of *FMRI*, leading to a decrease or absence of FMRP, the protein product of *FMRI*. FMRP is an mRNA-binding protein that is expressed ubiquitously and abundantly in neurons and glia during development, postnatally and in adult brain. FMRP is widely believed to participate in neurite morphogenesis, dendritic spine density forma-

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tion, and synaptic plasticity [3–7]. Our understanding of the pathogenesis of this disorder has increased significantly since the generation of the mouse model, the *Fmr1* knockout (KO) mouse, which exhibits many characteristics that resemble abnormal behaviors in humans with FXS such as hyperactivity, anxiety, and increased sensitivity to auditory stimuli [8–10]. Recently, novel targets for treatment of FXS have been developed, many of which are based on the ‘mGluR theory’ which posits that disrupted metabotropic glutamate receptor 5 synaptic plasticity underlies many of the FXS phenotypes. This theory, in large part, has been validated by pharmacological and genetic approaches in *Fmr1* KO mice, which have led to novel therapeutic approaches for fragile X, which pharmacologically target mGluR signaling [11, 12].

In addition to alterations in glutamatergic transmission, mounting evidence from studies of *Fmr1* KO mice indicate alterations in the expression of several important presynaptic and postsynaptic components of the inhibitory transmission machinery, including GABA_A receptor subunits, GABA transporters, glutamic acid decarboxylase, and gephyrin in different brain regions [13–18]. This evidence has led to a complementary ‘GABA theory’ [19], which argues that targeting the GABAergic system may also be an important approach for treatment in FXS. This approach is supported by studies in the *Drosophila* model of FXS, which have identified three molecules implicated in the GABAergic inhibitory pathway that were able to rescue several known *fmr1* mutant phenotypes, including mushroom body defects, excess Futsch translation and abnormal male courtship behavior [20]. In addition, it has been reported that taurine, a GABA_A receptor agonist, has been shown to improve cognitive functions in a mouse model of FXS [21]. We have recently reported that THIP, a superagonist at δ -subunit-containing presynaptic and extrasynaptic GABA_A receptors that mediates strong tonic inhibitory conductance in the CNS [13, 22], restored principal neuron excitability in amygdala slices from *Fmr1* KO mice to wild-type (WT) levels [18]. This suggested that acute treatment of THIP might also restore specific behavioral abnormalities in *Fmr1* KO mice. Previous studies have consistently shown pronounced hyperactivity, reduced retention of cued and spatial fear, and increased prepulse inhibition (PPI) in *Fmr1* KO mice [9, 23–25]. Consistent with previous studies, we reveal that these behavioral paradigms are similarly altered in *Fmr1* KO mice. Importantly, extending this work, our study shows that acute THIP treatment in *Fmr1* KO mice reverses specific key behavioral abnormalities, and thus supports the view

that enhancing GABAergic transmission via tonic inhibition might be a reliable way to ameliorate some core symptoms in FXS.

Materials and Methods

Animal Usage and Housing

Male *Fmr1* WT (stock No. 4828) and *Fmr1* KO (stock No. 4624) mice were on the congenic FVB mouse background and obtained from The Jackson Laboratory (Bar Harbor, Me., USA). For these studies, 3-month-old mice were used. Mice were maintained according to protocols approved by the Georgetown University Animal Care and Use Committee. Standard rodent chow and water were available ad libitum. The colony room was maintained on a 12-hour light/12-hour dark cycle at a temperature of 20°C and 55% humidity.

THIP was dissolved in sterile saline (0.89%), and injected intraperitoneally 30 min prior to testing at a volume of 10 ml/kg. The final dose was 3 mg/kg.

Open Field Test

Locomotion was measured as the spontaneous activity of mice in a novel open square arena (2 × 2 ft) brightly lit with overhead lights. Mice were tracked using the Topscan tracking system (Clever Sys Inc., Va., USA), as previously described [26]. Mice were tracked for 30 min, and the distance travelled and velocity were electronically recorded. We also analyzed the time spent in the center of the maze.

Fear Conditioning

Fear conditioning is a behavioral test in which mice learn to anticipate an adverse event. In our contextual fear conditioning paradigm, mice learn to associate a spatial environment (the apparatus) with the adverse event (footshock). For cued fear conditioning, mice were trained to associate an auditory cue with the adverse event. Cued fear conditioning is dependent on a functioning amygdala, but contextual fear conditioning also involves the hippocampus.

Acclimatization. On the day prior to training, the mice were acclimated to the test chamber for 8 min. The chamber consists of a clear Plexiglass 10 × 8-inch rectangular cage with a grid floor (Clever Sys Inc.). This is housed in a slightly larger box containing a house light, speaker and compact digital camera. The chamber was cleaned between animals using 95% ethanol.

Training. The training day involved returning the animals to the test chamber and exposing them to three consecutive training protocols consisting of a 2-min exploration followed by a 30-second nonaversive tone (the conditioned stimulus), followed immediately by a continuous 0.40-mA shock (the unconditioned stimulus) for 1 s. There was an additional rest period after the final training session before the mice were returned to their home cage.

Contextual Test. Twenty-four hours after training, the animals were scored for contextual fear by returning them to the exact same training chamber and observing them for freezing over a 5-min period. There was no conditioned stimulus or footshock.

Cued Test. Two hours after the contextual test, the mice were placed back in the chamber. To remove the contextual cues, the inside of the cage was altered. The grid floor was covered with a white plastic insert. The walls were covered with a nonporous,

Fig. 1. THIP prevents hyperactivity in *Fmr1* KO mice. Distance travelled (**a**) and average velocity (**b**) of WT and *Fmr1* KO mice in an open field test. Mice were tracked using a video-monitoring system over a 30-min period. * $p < 0.05$ vs. WT control; # $p < 0.05$ vs. *Fmr1* KO control. ANOVA and Newman Keuls post hoc. $n = 8$ per group.

orange, foam/plastic insert with a curved roof. To remove olfactory cues, the cage was wiped with a disinfectant instead of alcohol, and further wiped with water to reduce the odor. The mice were given 2 min for free exploration before presentation of a 30-second conditioned stimulus, as above. Freezing was measured from the start of the stimulus for a 2-min interval.

Auditory Startle Testing and PPI

The acoustic startle response (ASR) is typified by a flinching response to an auditory stimulus. The startle response can be reduced when the auditory stimulus is immediately preceded by a weaker stimulus, a process known as PPI. ASR and PPI were measured using the SR-LAB startle response system (San Diego Instruments, San Diego, Calif., USA), as previously reported [27]. The soundproof chamber ($16 \times 15 \times 23$) houses a Plexiglass mouse chamber (3.8 cm diameter) placed on a motion-sensitive platform. Two speakers are in the chamber, one that generates background white noise (65 dB), and the other transmitting the signal. The session was initiated with a 5-min acclimatization period followed by 5 presentations of the acoustic startle pulse alone (110 dB for 40 ms), which were excluded from the statistical analysis. This was followed by the random presentation of either the pulse alone, or the pulse with a 10-ms 6-, 9-, 12-, 15- or 18-dB prepulse above background stimuli (so prepulses were 71, 74, 77, 80 or 83 dB). This prepulse was presented 50 ms prior to the pulse. Each startle or prepulse was presented 3 times in a randomized fashion. The intertest interval was set to between 15 and 30 s and the trials were conducted with the house lights on. Startle response was recorded every millisecond for 65 ms following the onset of acoustic startle pulse. Maximal peak-to-peak amplitude was used to determine the ASR in the acoustic startle pulse and/or prepulse alone trials. PPI is expressed as percentage inhibition of the basal startle.

Results

Open Field Test

Spontaneous activity of WT and *Fmr1* KO mice was measured in the open field test. Animals were evaluated on distance travelled in a 30-min period and their average velocity during that time period. Mice were also evalu-

ated on the percentage of time they spent in the inner area of the arena versus the wall areas.

We found that *Fmr1* KO mice travelled significantly further than WT mice (increase of 32%, $p < 0.05$; fig. 1a), and had a greater average velocity (80 vs. 61 mm/s, $p < 0.05$; fig. 1b). These findings suggest that *Fmr1* KO mice on an FVB background exhibit hyperactive behavior, consistent with previous reports of mice on this background [9]. No differences were observed across genotypes regarding the percent of time the mice spent in the center of the maze, a measure sensitive to anxiety levels (WT: $13.6 \pm 1.8\%$, *Fmr1* KO: $15.3 \pm 2.2\%$).

THIP-treated mice also underwent analysis, and as sedation is an important factor when using GABA_A agonists, we carefully monitored injected mice. At the dose used (3 mg/kg), we saw no indication of sedation in either the WT or *Fmr1* KO mice, and THIP did not cause any effect on distance travelled or velocity in WT mice (fig. 1a, b). In contrast, THIP administration in *Fmr1* KO mice significantly attenuated both the distance travelled and velocity to WT levels ($p < 0.05$ compared to *Fmr1* KO mice baseline; fig. 1a, b). This finding demonstrates that THIP rescues hyperactivity in *Fmr1* KO mice.

Contextual and Cued Fear Conditioning

To determine if the absence of FMRP has an effect on acquisition of the task, freezing behavior was recorded in the 2-min intervals immediately following the tone and shock. Both *Fmr1* KO and WT mice had identical freezing patterns indicating that FMRP is not required for learning this task (fig. 2a). The mice were tested for contextual fear 24 h after training, and both *Fmr1* KO and WT mice displayed identical freezing, remaining immobile for approximately 36% of the trial period (fig. 2b). Two hours after the contextual test, we probed for the

Fig. 2. *Fmr1* KO mice have decreased cued, but not contextual, fear conditioning. **a** Acquisition of conditioned fear in WT and *Fmr1* KO mice. Freeze time was measured as a percent of the trial block (2 min). Three shocks were presented to the mice followed by a 2-min interval. **b** Percent freeze time in contextual and cued testing 24–26 h after training. * $p < 0.05$, unpaired t test. **c** The effects of THIP administration 30 min prior to cued fear testing. * $p < 0.05$ vs. *Fmr1* KO (control). ANOVA and Newman Keuls post hoc. $n = 8$ per group.

cued response. The mice were placed in a chamber with altered walls, floors, ceiling shape and odor. After a 2-min exploration, they were presented with the same auditory cue that preceded the footshock in the training period. Freezing behavior was measured, and WT mice froze for 25% of the trial period compared to 9% for *Fmr1* KO mice, a statistically significant difference ($p < 0.05$; fig. 2b).

We repeated our experiments, but this time included THIP treatment 30 min prior to the cued fear test. Mice were treated with THIP 30 min prior to entering the novel arena. Interestingly, while THIP administration significantly reduced freezing in the WT mice, it did not alter the low freezing response in *Fmr1* KO mice (fig. 2c; $p < 0.01$), indicating that enhancing tonic GABAergic transmission via THIP does not have an effect on cued fear conditioning in *Fmr1* KO mice.

Auditory Startle Testing and PPI

Auditory startle and PPI are measures of sensorimotor gating, which is the ability of an animal to integrate sensory information. The response to auditory startle was significantly impaired in *Fmr1* KO mice, being reduced by 65% ($p < 0.01$; fig. 3a). PPI in *Fmr1* KO mice was significantly increased with all prepulses, but was more pro-

nounced with lower decibel prepulses [287% increase (6 dB), 72% (9 dB), 72% (12 dB), 61% (15 dB), 56% (18 dB); $p < 0.01$ for all prepulses; fig. 3b].

THIP treatment did not alter the startle response in *Fmr1* KO mice, with treated mice having a 58% decrease in startle size (fig. 3a). While THIP treatment also had no effect on PPI in WT mice, THIP did cause changes in PPI in *Fmr1* KO mice (fig. 3b). At low prestimulus intensity (6 dB above background), there was attenuation of PPI in THIP-treated mice compared to controls ($p < 0.05$; fig. 3b). As the prestimulus was increased to 74 dB (9 dB above background), THIP prevented the significant difference between WT and *Fmr1* KO mice (fig. 3b). As the prepulse volume increased above 74 dB, the effect of THIP disappeared.

To enable a proportional comparison between control and THIP-treated mice, we converted the PPI data to percent WT (comparing control-WT to control-KO, and THIP-WT to THIP-KO). Using this scale, the effect of THIP treatment at low decibel prepulses becomes apparent. At 6 dB, *Fmr1* KO mice have a 287% increase in PPI, but *Fmr1* KO THIP-treated mice only have a 109% increase (178% reduction; $p < 0.01$; fig. 3c). When the prepulse was 9 dB above background, *Fmr1* KO mice have a 72% higher

Fig. 3. THIP attenuates the *Fmr1* KO-induced increase in PPI. **a** The startle response of WT and *Fmr1* KO mice to a 120-dB white noise pulse is not affected by THIP treatment 30 min prior to entering the sound chamber. **b** PPI is significantly increased in *Fmr1* KO mice compared to WT controls. The greatest difference between genotypes is observed with the lower decibel prepulses. This effect is blunted in THIP-treated mice, but only at low decibel prepulses. The x-axis displays prepulse in dB above background (65 dB). ** $p < 0.01$; *** $p < 0.001$; n.s. = non-significant vs. WT (control). # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$; n.s. = non-significant vs. WT (THIP). ANOVA with Newman Keuls post hoc (performed individually for each prepulse volume). **c** Change in PPI in *Fmr1* KO mice as a percentage of WT mice. WT mouse response was set at 100%. THIP-treated *Fmr1* KO mice were compared to THIP-treated WT mice. *Fmr1* KO control mice were compared to WT control mice. * $p < 0.05$; ** $p < 0.01$, unpaired t test. *Fmr1* KO (control) vs. *Fmr1* KO (THIP). $n = 8$ per group.

PPI, but THIP-treated *Fmr1* KO mice only have a 21% increase in PPI (a 51% reduction; $p < 0.05$; fig. 3c). As the prepulse volume increased, the attenuation of PPI by THIP decreased compared to WT. These data confirm earlier findings that *Fmr1* KO mice have different sensitivities to audible cues depending on the volume [25, 28], and demonstrate that THIP can partially reverse these deficits.

Discussion

Development of potentially effective treatments in humans for FXS relies on understanding the underlying brain defects as well as being able to genetically and/or pharmacologically correct synaptic, behavioral and cog-

nitive abnormalities in the *Fmr1* KO mouse model. In the present study, we provide evidence that treatment with THIP, a GABA_A agonist which acts upon tonic GABA receptors, attenuates the motor hyperactivity and sensory hypersensitivity in *Fmr1* KO mice. As THIP is not of the typical benzodiazepine class of GABA agonists, this suggests that tonic inhibition may be a major target for drug therapy and furthers the concept that GABA_A receptors are a viable therapeutic target for this disorder as previously postulated [19].

Mounting evidence implicates the GABAergic system, and specifically the tonic form of inhibition, as a potential therapeutic target for developing novel treatments for other brain disorders such as epilepsy, premenstrual dysphoric disorder and stroke [22, 29–31]. In relation to FXS,

it has been reported that GABA exposure during development can rescue the biochemical, morphological, and behavioral phenotypes of the *Drosophila* model of FXS [20], and taurine, a GABA_A receptor agonist, improves cognitive functions in a mouse model of FXS [21]. In this study, we have used THIP, a superagonist at δ -subunit-containing perisynaptic and extrasynaptic GABA_A receptors, and as such has its strongest effects on tonic inhibition [32]. Interestingly, direct binding between FMRP and the mRNA of the δ -subunit of the GABA_A receptor has been shown in *Fmr1* KO mice [33]. In addition, expression of this subunit is disrupted with a reduction of protein levels in *Fmr1* KO mice [16], consistent with a previous study showing downregulation of tonic GABAergic inhibition in the subiculum [15], supporting the concept that tonic inhibition is defective in *Fmr1* KO mice. Indeed, we have recently shown that tonic inhibitory currents are decreased in the amygdala in *Fmr1* KO mice, and bath application of THIP rescues neuronal hyperexcitability in the amygdala of *Fmr1* KO slice cultures [18]. Consistent with this, here we provide evidence that THIP can correct specific behavioral abnormalities in *Fmr1* KO mice that are relevant to the human condition.

Hyperactivity is one of the often-cited behavioral abnormalities in FXS [34, 35], and has also been previously reported in *Fmr1* KO mice on the FVB background [9, 36, 37]. In this study, we report the presence of this hyperactivity, and its susceptibility to THIP administration. We believe that this dampening of hyperactivity is not due to sedative effects of THIP as 3 mg/kg had no effect on ambulation or velocity in control mice.

Methylphenidate, a psychostimulant drug commonly used to modify hyperarousal and hyperactivity in FXS, which mainly targets the dopamine system, also targets the noradrenergic system. Methylphenidate appears to enhance inhibitory synaptic transmission in the locus ceruleus in juvenile rats [38]. Since it has been reported that local GABAergic interneurons might play a role gating locus ceruleus function [39], the major brain region for noradrenergic transmission, it is intriguing to speculate that GABA_A receptors are downregulated in the locus ceruleus, as has been reported in several other brain regions in FXS [16]. Therefore, enhancing GABAergic transmission might restore some degree of inhibition onto noradrenergic cells, therefore lowering hyperactivity in *Fmr1* KO mice.

Many groups, with variable results, have studied the behavioral phenotype of the *Fmr1* KO mouse with regard to fear conditioning. The background strain appears to be an important factor in the appearance of many of these

phenotypes [24, 40, 41], and even amongst laboratories using a similar background strain the results remain inconsistent. For example, in the present study, we report that cued, but not contextual, fear conditioning is altered in *Fmr1* KO mice, while Paradee et al. [24] reported a decrease in both contextual and cued freezing. Given our previous results on the effects of THIP on excitability in the amygdala of *Fmr1* KO mice [18], we were very interested in examining the effect of THIP on *Fmr1* KO mice behavior in amygdala-dependent behavioral paradigms. However, we found that THIP administration 30 min prior to the recall test could not ameliorate the behavioral deficit observed in *Fmr1* KO mice. Instead we found that it significantly decreased the freezing time of control mice. This effect is probably due to the anxiolytic effect of GABA_A agonists that has previously been reported [42]. As *Fmr1* KO mice were already at the lower end of the freezing detection scale, this effect was not seen in *Fmr1* KO mice. Taking together, our results and similar studies in cued fear conditioning [24] as well as physiological studies showing decreased long-term potentiation in the lateral amygdala [43, 44] suggest that *Fmr1* KO mice have amygdala-based learning and memory deficits regarding the cued auditory fear conditioning. This may explain why *Fmr1* KO mice have diminished fear responses such as freezing behavior. Increasing evidence supports that inhibition within the amygdala plays a central role in gating pre- and postsynaptic plasticity in the lateral amygdala, the major nucleus receiving sensory input from different brain regions, and therefore in fear-related behaviors (review in Ehrlich et al. [59]). We have recently described a profound defective GABAergic transmission in the basolateral nucleus of the amygdala [18], but to our knowledge there is not a single study focusing on GABAergic transmission within the lateral amygdala in *Fmr1* KO mice. Since decreasing GABAergic transmission can improve learning or retrieval of conditioned fear memories and impairs extinction memory retrieval in a context-specific manner [45–47], it might be possible that GABAergic transmission is enhanced in the lateral amygdala, and reducing GABAergic transmission might have different outcomes in this behavioral paradigm.

Another core problem in individuals with FXS is the increase in sensitivity to auditory stimuli [48–50]. This hypersensitivity can be reproduced in *Fmr1* KO mice as abnormal ASR and PPI responses. Increased excitability in the ascending auditory pathway due to reduced GABA_A receptor-mediated inhibition has been postulated as a possible mechanism for diminished startle response in *Fmr1* KO mice [51]. Nevertheless, our re-

sults seem to rule out this possibility since enhancing GABAergic transmission with THIP treatment did not alter the startle response in *Fmr1* KO mice. Furthermore, studies have shown that *Fmr1* KO mice have increased PPI of auditory startle [25, 28]. Nielsen et al. [25] found that *Fmr1* KO mice on a C57/Bl6 background had a significant elevation in PPI when presented with a prepulse 2 dB above background, but not a prepulse 4 or 8 dB above background. Chen and Toth [28] used *Fmr1* KO mice on an FVB background and found that a prepulse 10 dB above background enhanced the PPI by 96% compared to control mice, but only by 56% at 20 dB above background. In the present study, we found that *Fmr1* KO mice on the FVB background had a 287% increase in PPI at 6 dB, but this could be volume dependently decreased to 72% at 9 dB and to 56% at 18 dB. These changes in PPI could be due to the increased sensitivity of *Fmr1* KO mice to low-intensity bursts, causing an increased PPI at low prepulses. THIP dampened the elevation in the PPI response back towards control levels; however, it only had an effect in the low-intensity prepulse window. The greatest effect was seen with the 6-dB prepulse, where THIP reduced the PPI by 128%. A strong effect was also observed when using the 9-dB prepulse, as THIP reduced PPI by 51%. As the volume of the prepulse increased, the effect of THIP was lost. One possible explanation for this partial restoration is that it is known that PPI is regulated by many neurotransmitter systems, including the GABAergic system [52, 53], making highly complex a complete restoration of PPI targeting only one of the systems involved. Furthermore, one study in *Fmr1* KO mice has shown decreased PPI of startle eyelid responses with the magnetic distance measurement [23]. This correlates with findings in humans with FXS [54, 55], and thus supports a role for FMRP in sensorimotor processing both in humans and mice. Interestingly, recent studies using THIP, which we have shown to have amygdala effects [18], have also demonstrated the existence of a potent tonic

inhibitory neurotransmission in the rat auditory thalamus [56] and in the avian nucleus laminaris [57], suggesting an active role of tonic inhibition in modulating auditory processing cues.

In conclusion, we have demonstrated that *Fmr1* KO mice have behavioral deficits in numerous behavioral paradigms, and that administration of the tonic GABA_A receptor agonist THIP can ameliorate some of these core defects. Thus, these data extend our previous results showing that THIP rescues core electrophysiological deficits in *Fmr1* KO neurons [18], and strongly supports the hypothesis that enhancing tonic inhibition may be a putative target for the treatment of FXS. These data are especially intriguing as recent very promising phase II clinical trials in FXS indicate that GABA_B receptor agonists such as R-Baclofen (STX209, Seaside Therapeutics) have a positive outcome for amygdala-based symptoms such as social avoidance. Interestingly, a clinical trial is already scheduled to study the effect of ganaxolone, a neurosteroid with high affinity for the δ -subunit of GABA_A receptors, in children with FXS at the MIND Institute of the University of California at Davis [58]. Thus, it is intriguing to speculate that the δ -subunit of GABA_A receptors might be a promising target for treatment in FXS. Indeed, based on the results presented here combined with our previous study with THIP [18], we predict that this trial will also have a positive outcome for amelioration of amygdala-based and nonamygdala-based symptoms in FXS.

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