THERAPEUTIC EFFICACY OF THE BKCA CHANNEL OPENER CHLORZOXAZONE IN A MOUSE MODEL OF FRAGILE X SYNDROME

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ABSTRACT

Fragile X syndrome (FXS) is an X-linked neurodevelopmental disorder characterized by several behavioral abnormalities, including hyperactivity, anxiety, sensory hyper-responsiveness and autistic-like symptoms such as social deficits. Despite considerable efforts, effective pharmacological treatments are still lacking, prompting the need for exploring the therapeutic value of existing drugs beyond their original approved use. One such repurposed drug is chlorzoxazone which is classified as a large-conductance calcium-dependent potassium (BKCa) channel opener. Reduced BKCa channel functionality has been reported in FXS patients, suggesting that molecules activating these channels could serve as promising treatments for this syndrome. Here, we sought to characterize the therapeutic potential of chlorzoxazone using the *Fmr1*-KO mouse model of FXS which recapitulates the main phenotypes of FXS, including BKCa channel alterations. Chlorzoxazone, administered either acutely or chronically, rescued hyperactivity and acoustic hyper-responsiveness as well as impaired social interactions exhibited by *Fmr1*-KO mice. Chlorzoxazone was more efficacious in alleviating these phenotypes than gaboxadol and metformin, two repurposed treatments for FXS which do not target BKCa channels. Systemic administration of chlorzoxazone modulated the neuronal activity-dependent gene *c-fos* in selected brain areas of *Fmr1*-KO mice, corrected aberrant hippocampal dendritic spines and was able to rescue impaired BKCa currents recorded from hippocampal and cortical neurons of these mutants. Collectively, these findings provide further preclinical support for BKCa channels as a valuable therapeutic target for treating FXS and encourage the repurposing of chlorzoxazone for clinical applications in FXS and other related neurodevelopmental diseases.

INTRODUCTION

Fragile X Syndrome (FXS) is the most common form of inherited intellectual disability and the leading monogenic cause of Autism Spectrum Disorder (ASD). It is caused by a mutation in the Fragile X Mental Retardation 1 (*FMR1*) gene located on the X chromosome, resulting in a deficiency of the Fragile X Mental Retardation Protein (FMRP) [1]. This RNA-binding protein regulates dendritic branching, synaptic function, and neuronal maturation [2, 3]. Its absence induces physical anomalies (e.g., facial elongation, macro-orchidism) and several behavioral abnormalities, including hyperactivity, sensory hyper-responsiveness, anxiety and cognitive deficits, often associated with typical autistic symptoms such as impaired social interactions [4].

Despite considerable advances in the understanding of the pathophysiology of FXS, there is to date no cure for this neurodevelopmental disease. None of the newly designed therapeutic compounds tested clinically were successful in rescuing the full array of neurobehavioral symptoms associated with FXS, with most pharmacological approaches targeting only a limited set of symptoms (e.g., anxiolytics or antipsychotics for hyperactivity) [5-8]. These treatments not only have limited efficacy, but may also induce side effects that are particularly undesirable due to the developmental nature of this syndrome. New therapies are therefore urgently needed for FXS. As a result, there has been a concerted effort to repurpose several existing molecules for FXS treatment [6, 7, 9]. Among these, encouraging preclinical results were obtained with metformin [10], a first-line therapy for type 2 diabetes which normalizes matrix metallo-proteinase 9 (MMP9) whose expression was reported to be elevated in the brains of FX patients and the *Fmr1* knock-out (KO) mouse model for the syndrome [11]. The observation of a deficient GABAergic tonic inhibition in these mice have also prompted studies involving selective $GABA_A$ receptor agonists such as gaboxadol [12, 13], which was initially considered for its analgesic and anxiolytic functions and for treating insomnia [14, 15]. However, the acute and chronic efficacy of these treatments was not always convincingly demonstrated on multiple behavioral phenotypes expressed in the *Fmr1*-KO mouse model of FXS [10, 13].

Big-conductance Ca^{2+}-activated K^+ (BKCa) channels, which modulate membrane excitability, intracellular Ca^{2+} homeostasis and neurotransmitter release [16-20], have emerged as another potentially valuable therapeutic target due to their direct interaction

with FMRP signalling which controls their expression and function in hippocampal and cortical excitatory neurons [21-23]. Notably, the lack of FMRP reduces neuronal BKCa channel activity [22, 24] and leads to neuronal hyper-excitability [25]. Furthermore, mutations in the *FMR1* gene selectively disrupting the interactions between FMRP and BKCa channels are sufficient to induce selected major FXS phenotypes in humans [26]. Moreover, BKCa channel activity is regulated by a broad spectrum of kinases, including CaMKII [27, 28], which is a well-known target of FMRP [29]. Importantly, the expression and functionality of BKCa channels are markedly reduced in the lymphoblasts of FXS patients and in the brains of *Fmr1*-KO mice [24], which might be responsible for their altered neurotransmitter release and sensory hypersensitivity [25, 30]. Altogether, these observations support BKCa channel openers as a promising therapeutic strategy to rescue FXS-like neurobehavioral phenotypes.

Chlorzoxazone (5-chloro-2,3-dihydro-1,3-benzoxazol-2-one; CHLOR), a FDA-approved drug, is classified as a skeletal muscle relaxant [31] which exerts its effects primarily at the level of the spinal cord and subcortical areas of the brain [32]. Recently, CHLOR has emerged as a brand-new value in healthcare [33], exerting immunomodulatory [34] and neuroprotective effects in preclinical mouse studies [35-39]. Due to its BKCa activating properties [40], we sought to evaluate the potential of CHLOR as a novel repurposed treatment for FXS. To this end, experiments were designed to provide an extensive characterization of the effects of acute (a single dose) and chronic (daily dose for 10 days) systemic administration of CHLOR on multiple FXS-like behavioral phenotypes in the original first generation *Fmr1*-KO mouse model, including comparisons with other repurposed treatments for FXS, namely metformin (MET) and gaboxadol (GAB). This mouse line recapitulates most of the physical and behavioral abnormalities observed in FXS patients [41-44] and has been also most largely used to identify therapeutic approaches for FXS (reviewed in [45]). However, since the *Fmr1* promoter remains intact in these transgenic mice, transcription remains possible, resulting in abnormal residual *Fmr1* RNAs [46, 47]. To circumvent this issue, a complete *Fmr1* null mouse was then developed, characterized by the absence of all *Fmr1* mRNA transcripts [46]. Although the behavioral FXS-like phenotype in this "second generation" model of FXS—also known as *Fmr1*-KO2 [46]—seems less robust compared to the first generation *Fmr1*-KO model [48], we also evaluated the replicability of the behavioral effects of CHLOR in the *Fmr1*-KO2 mouse model. Finally, to gain further functional insights into how the systemic administration of CHLOR modulates the neuronal

circuitry, we examined its dose-dependent effects on the expression of the neuronal activity-dependent marker *c-fos*, the density and morphology of dendritic spines, as well as the patterns of neuronal BKCa currents in selected brain areas of *Fmr1*-KO mice and their WT littermates. Our findings uncover the benefits of CHLOR for treating FXS phenotypes and identify BKCa channels as a valuable target for treating neurodevelopmental disorders.

METHODS

As illustrated in Table S1, a total of 11 studies were performed, each employing different batches of mice. As commonly done in research studies on the *Fmr1*-KO model, only adult (4-8 month-old) males were used since they exhibit the most robust FXS- and ASD-like neurobehavioral phenotypes [45, 48-50]. The detailed description of the methods used for behavioral and brain assessment is provided in **Supplementary Material**. All experimental procedures were approved by the local ethical committee ("Comité d'Ethique pour l'expérimentation animale de Bordeaux", CE 50) and the French Ministry ("Ministère de l'enseignement supérieur de la recherche et de l'innovation", authorization APAFIS#29546) and followed the ARRIVE guidelines [51].

All data were analyzed with ANOVA using treatment and genotype as the between-subject factors and stimulus intensity as the within-subject factor for the startle data. Post-hoc comparisons (Tukey's-Kramer test) were performed when a significant interaction genotype x treatment was found. Otherwise, separate one-way ANOVAs in each treatment group with genotype as the between subject factor were conducted, if appropriate. Normality was assessed through the Shapiro-Wilks test; data from startle reactivity were subjected to natural logarithmic transformation in order to meet the normality requirements of ANOVA. All analyses were carried out using Statview and PASW Statistics 18.

RESULTS

Chlorzoxazone rescues FXS-like behavioral phenotypes in *Fmr1*-KO mice

To select the appropriate dose of CHLOR to be tested in *Fmr1*-KO mice, we first determined the optimal dose of CHLOR following systemic administration of ascending doses from 5 to 20 mg/kg in C57BL/6J mice, i.e., the same genetic background as *Fmr1*-KOs. This behaviorally relevant dose was defined as the highest dose that would not induce significant changes in locomotor activity or acoustic startle response, i.e., two behavioral domains which are

typically impaired in *Fmr1*-KO mice. Results from this pilot study showed that activity in the open field was affected in a dose-dependent manner, with the 10 mg/kg dose of CHLOR starting to decrease exploration, indicative of potential adverse effects (treatment effect: $F_{3,34} = 6.12$, $p = 0.002$; **Fig. S1A**). Doses of CHLOR ranging from 5 mg/kg and above did not alter the acoustic startle response (treatment effect and treatment x stimulus intensity interaction: $F_{3,34} = 0.57$, and $F_{9,102} = 1.24$, NS; **Fig. S1B)**. We therefore selected a maximal dose of 5 mg/kg for subsequent dose-dependent studies involving *Fmr1*-KO mice.

Fmr1-KO mice injected with vehicle displayed a clear hyperactivity in the open field (genotype effect: $F_{1,69} = 74.59$, $p < 0.0001$; **Fig. 1A**) and an acoustic hyper-responsiveness in the startle test that increased with stimulus intensity (genotype effect and its interaction with intensity: $F_{1,14} = 13.56$ and $F_{3,204} = 6.79$; $p = 0.025$ and 0.0002; **Fig. 1B**). CHLOR dose-dependently rescued these two phenotypes. While the two highest doses (i.e. 3.75 and 5 mg/kg) were efficacious in the open field (interaction genotype x treatment: $F_{4,68} = 10.13$, $p < 0.0001$; **Fig. 1A**), only the highest dose of 5 mg/kg normalized startle reactivity of *Fmr1*-KO mice [although the interaction genotype x treatment failed to reach statistical significance ($F_{4,68} = 2.26$, $p = 0.07$), significant genotype effects were found at all CHLOR doses (1.25 mg/kg: $F_{1,14} = 4.76$, $p = 0.047$; 2.5 mg/kg: $F_{1,14} = 8.44$, $p = 0.012$; 3.75 mg/kg: $F_{1,13} = 9.14$, $p = 0.01$), except for the 5 mg/kg ($F_{1,13} = 0.11$, NS; **Fig. 1B**)]. Furthermore, no significant treatment effect emerged in WT mice on any considered behavioral variable, as demonstrated by post-hoc comparisons on total distance moved (**Fig. 1A**) and separate one-way ANOVAs on startle response (treatment effect in WT mice: $F_{4,30} = 1.2$, NS; in KOs: $F_{4,38} = 2.62$, $p = 0.049$, with a significant difference between KO-VEH and KO-CHLOR 5, **Fig. 1B**). Hence, we administered CHLOR at the dose of 5 mg/kg for all the remaining studies.

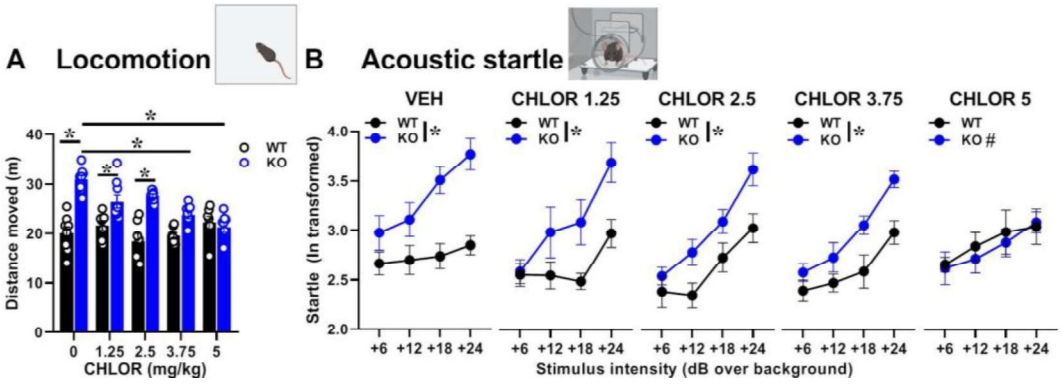

Fig. 1: **Dose-dependent acute effects of chlorzoxazone (CHLOR) in *Fmr1*-KO mice.** The efficacy of the optimal dose of 5 mg/kg and the therapeutic potential of lower doses of CHLOR were assessed in the open field (A) followed by the acoustic startle (B) tests. N = 9-10; *p < 0.05. # p < 0.05 versus KO-VEH from separate ANOVAs.

VEH=vehicle. Doses are expressed as mg/kg; acute treatments were administered i.p. 1 h before testing. Stimulus intensities are expressed over (+) a background of 66dB. Startle reactivity data were submitted to natural logarithmic (ln) transformation in order to meet the normality criteria of ANOVA. Data are mean ±SEM.

We further explored whether these acute behavioral effects of CHLOR could be generalized using the second generation *Fmr1*-KO2 model that has mostly been employed for brain investigations [25]. As for the first generation model, vehicle-injected *Fmr1*-KO2 mice exhibited hyperactivity in the open field and an exaggerated startle response (genotype effects: $F_{1,24}$ = 6.02 and 16.07, p = 0.02 and 0.0005, respectively; **Fig. S2A**, **C**). In agreement with previous reports [48], social interaction was unaltered in these *Fmr1*-KO2 mutants (genotype effect: $F_{1,23}$ = 0.27, NS; **Fig. S2B**). Acute administration of CHLOR at the dose of 5 mg/kg was able to rescue both locomotor (interaction genotype x treatment: $F_{1,27}$ = 13.86, p = 0.04) and acoustic phenotypes in these mutants (interaction genotype x treatment: $F_{1,24}$ = 4.45, p = 0.04; **Fig. S2A**, **C**), without exerting significant effects in WT mice (non-significant post-hoc tests).

Acute and chronic efficacy of chlorzoxazone: comparisons with gaboxadol and metformin

To gain further insights into the relative efficacy of CHLOR, we compared its behavioral signature to that of two other repurposing drugs proposed to treat FXS, the gabaergic agonist gaboxadol and the glycemic regulator metformin. In a first set of experiments, *Fmr1*-KO mice were treated acutely with these compounds one hour before undergoing a behavioral test battery assessing locomotor activity, social interaction and the acoustic startle response (**Fig. 2**). Confirming our previous results [24, 44, 52-54], *Fmr1*-KO mice treated with vehicle showed hyperactivity and reduced social interaction (**Fig. 2A, B**). These abnormalities were fully rescued by CHLOR at the dose of 5 mg/kg; as for gaboxadol, there was a tendency towards an attenuation of these behavioral alterations in mutant mice, post-hoc comparisons between KO-VEH and KO-GAB groups failing to reach statistical significance (genotype x treatment interaction for total distance traveled: $F_{3,64}$ = 4.52, p = 0.006, for social affiliation time: $F_{3,64}$ = 6.26, p = 0.0009; **Fig. 2A, B**). Similarly, the exacerbated acoustic startle response, especially at the highest noise intensity, of *Fmr1*-KO vehicle-treated mice was fully rescued by CHLOR (dose of 5 mg/kg), while only attenuated by gaboxadol in a dose-dependent manner (interaction genotype x treatment x stimulus intensity: $F_{3,192}$=3.94, p=0.0001; **Fig. 2C**). Although a tendency to an intensity-dependent increase in startle response emerged following CHLOR and GAB treatments in WT mice, no statistically

significant difference was confirmed by post-hoc comparisons between WT-VEH and WT-CHLOR or -GAB groups (**Fig. 2C**).

As to metformin, acute treatment at the dose of 200 mg/kg failed to attenuate the behavioral abnormalities of *Fmr1*-KO mice, contrasting with CHLOR which was again efficacious in rescuing all FXS phenotypes (genotype x treatment interaction for total distance traveled and affiliation time: $F_{2,53}$=6.13 and $F_{2,48}$=8.85, p=0.004 and p=0.0005; **Fig. 2D, E**), including startle hyper-responsiveness (interaction genotype x treatment x stimulus intensity: $F_{6,159}$=2.09, p=0.06; treatment effect: $F_{2,53}$=3.33, p=0.04; genotype effects and their interactions with stimulus intensity in separate ANOVAs: in VEH $F_{1,16}$=6.06 and $F_{3,48}$=8.14, p=0.03 and 0.0002; in MET $F_{1,19}$=5.66 and $F_{3,57}$=0.52, p=0.03 and NS, in CHLOR, $F_{1,18}$=0.24 and $F_{3,54}$=0.20, NS; **Fig. 2F**). Once again, no significant treatment effect emerged in WT mice on any behavioral variable (post-hoc comparisons, NS; **Fig. 2D, E**; treatment effect in separate ANOVA in WT mice: $F_{2,31}$ = 1.46, NS; **Fig. 2F**).

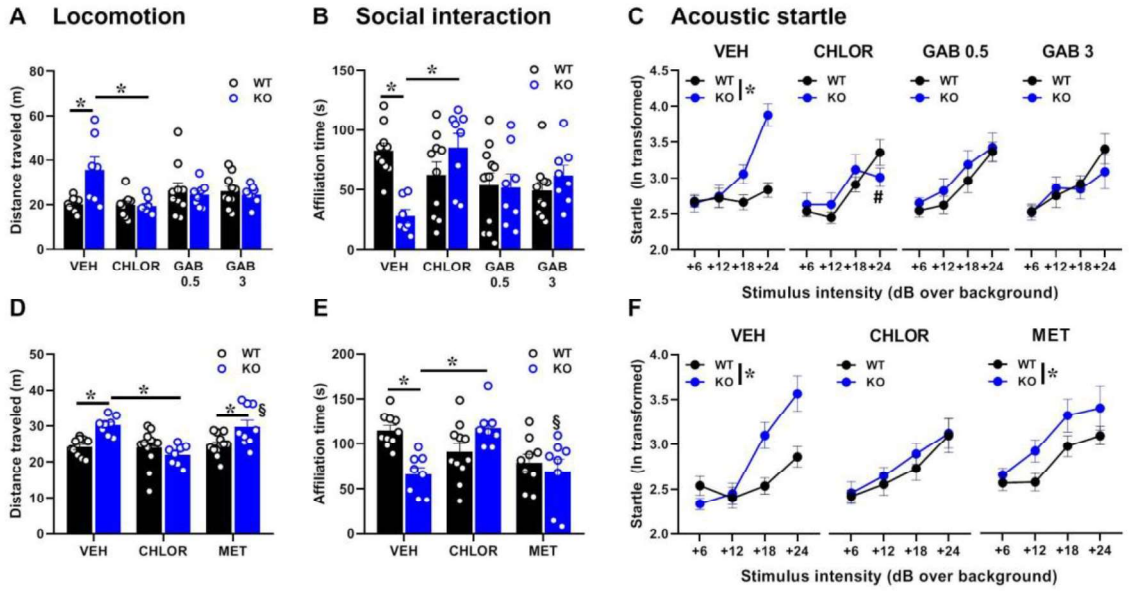

Fig. 2: Acute behavioral effects of chlorzoxazone (CHLOR, 5mg/Kg) compared with gaboxadol (GAB, 0.5 and 3 mg/kg) and metformin (MET, 200 mg/kg). Behavior was assessed in the open field (A, D), social interaction (B,E) and acoustic startle (C,F) tests as in previous studies. N =7-9 (A, B and C); N = 8-10 (D, E and F). *p < 0.05. # versus KO-VEH, § versus KO-CHLOR, p < 0.05 from post-hoc tests. Doses are expressed as mg/kg; all treatments were administered 1 h before testing. Stimulus intensities are expressed over (+) a background of 66 dB. VEH= vehicle. Data are mean ± SEM.

In a second set of experiments, we examined the behavioral effects of repeated systemic injections of CHLOR. When administered chronically for 10 days, CHLOR was efficacious in rescuing hyperactivity, reduced social interaction and exaggerated acoustic startle response shown by *Fmr1*-KO mutants (genotype x treatment interaction on total

distance and affiliation time: $F_{3,64}$=5.10 and 9.16, p=0.003 and <0.0001; **Fig. 3A, B**; genotype x treatment and genotype x treatment x stimulus intensity interactions on startle: $F_{3,56}$=3.36, $F_{9,168}$=2.87, p=0.025 and 0.004 **Fig. 3C**). Chronic CHLOR also attenuated the increased expression of stereotypical and anxiety-like self-grooming behavior of *Fmr1*-KO mice (genotype x treatment interaction on self-grooming time: $F_{3,64}$=5.67, p=0.002; **Fig. 3D**). Contrary to CHLOR, chronic administration of gaboxadol for 10 days at the dose of 0.5 mg/kg or 3 mg/kg failed to rescue all these abnormalities (**Fig. 3A-C**), although a non-significant trend towards attenuation was observable on self-grooming levels **(Fig.3-D)**. So did chronic administration of metformin at the dose of 200 mg/kg (genotype x treatment interaction on total distance, social affiliation and acoustic startle: $F_{2,34}$=7.05, $F_{2,30}$=5.32, $F_{2,34}$=4.48, p=0.003, 0.01, 0.02**; Fig. 3E, F and G**), with the exception of the anxiogenic self-grooming profile of *Fmr1*-KO mice on which MET exerted rescuing effects equivalent to CHLOR (genotype x treatment interaction on self-grooming time: $F_{2,30}$=8.24, p=0.0001; **Fig. 3H**).

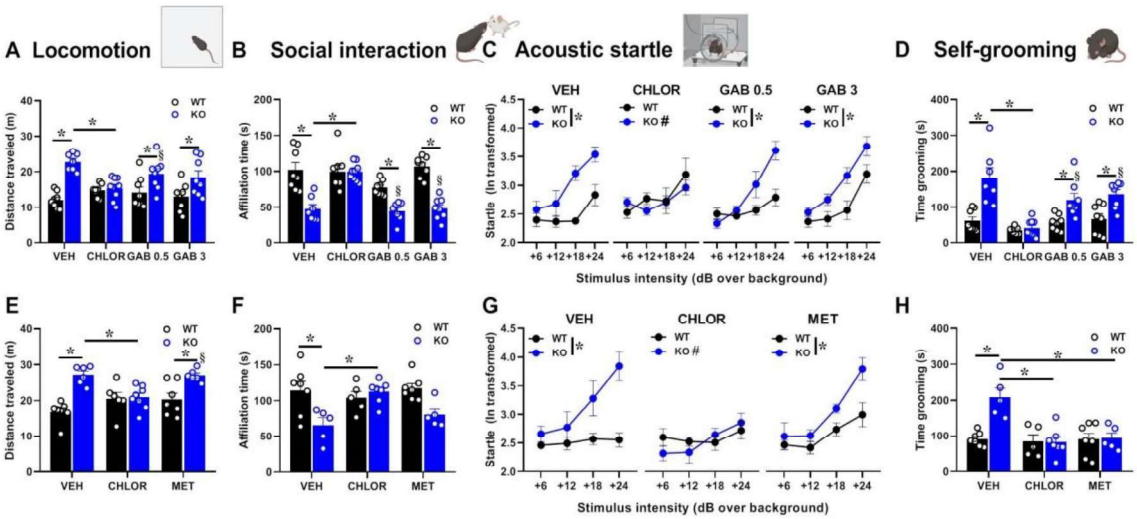

Fig. 3: Chronic behavioral effects of chlorzoxazone (CHLOR, 5mg/Kg) compared with gaboxadol (GAB, 0.5 and 3 mg/kg) and metformin (MET, 200 mg/kg). Behavior was assessed in the open field (A, E), social interaction (B, F), acoustic startle (C, G) and self-grooming (D, H) tests as in previous studies. N = 7-9 (A-D) and 6-8 (E-H). *p < 0.05. # versus KO-VEH, § versus KO-CHLOR, p < 0.05 from post-hoc tests. Doses are expressed as mg/kg. Treatments were administered i.p; once daily for 10 days before the beginning of behavioral testing (see also Table S1) and continued until completion of the testing battery. Stimulus intensities are expressed over (+) a background of 66 dB. VEH = vehicle. Data are mean ± SEM.

The body weight of all mice was measured upon completion of the test battery. Mouse testes were also dissected and weighed to assess the potential effects of these treatments on macro-orchidism, a physical phenotype typically exhibited by *Fmr1*-KO mice and reported in FXS patients [45, 55, 56]. While the 5 mg/kg dose of CHLOR marginally affected body weight loss compared to vehicle-treated mice (**Fig. S3A, C)**, the highest dose of

gaboxadol and the 200 mg/kg dose of metformin respectively reduced and enhanced weight loss in both WT and *Fmr1*-KO mice compared to VEH treatments (treatment effect for gaboxadol study: $F_{3,54} = 6.76$, $p = 0.0006$; **Fig. S3A**; treatment effect for metformin study: $F_{2,34} = 10.25$, $p = 0.0003$; **Fig. S3C**). The increased testicular volume of *Fmr1*-KO mice treated with vehicle (**Fig. S3B,D**) was unaffected by chronic CHLOR or gaboxadol administration (genotype effect: $F_{1,56} = 66.42$, $p<0.0001$; genotype x treatment: $F_{3,56} = 0.64$, NS; **Fig. S3B**). Metformin also failed to eliminate the macro-orchidism characterizing *Fmr1*-KO mice (genotype effect $F_{1,34} = 19.51$, $p<0.0001$; genotype x treatment interaction: $F_{2,34} = 0.94$, NS; **Fig. S3D**).

Effects of Chlorzoxazone on neuronal activity and structural plasticity in *Fmr1* mice

To identify brain areas targeted by an acute systemic injection of CHLOR in WT and *Fmr1*-KO mice, we performed cellular imaging of the activity-dependent gene *c-fos* classically used as an indirect marker of neuronal activity [57, 58]. Compared to vehicle-treated mice, the behaviorally active dose of 5 mg/kg of CHLOR, but also a higher dose of 10 mg/kg, reduced Fos expression in the hippocampal CA1 of *Fmr1*-KO mice, but not of WT littermates (genotype x treatment effect: $F_{2,116} = 21.66$, $p < 0.0001$; treatment effect: $F_{2,116} = 6.50$, $p = 0.002$; **Fig. 4A**). Such a pattern of CHLOR-induced neuronal hypoactivity restricted to *Fmr*1-KO mice was also observed in the dentate gyrus (genotype x treatment interaction: $F_{2,116} = 4.18$, $p = 0.02$; treatment effect: $F_{2,116} = 6.50$, $p = 0.002$; **Fig. 4C**), but not in the hippocampal CA3 (genotype x treatment interaction: $F_{2,116} = 0.10$, NS; **Fig. 4B**). In the prefrontal cortex, CHLOR also reduced Fos expression both in the prelimbic and infralimbic areas again only in *Fmr1*-KOs (genotype x treatment interaction: $F_{2,114} = 9.73$, $F_{2,116} = 4.32$, $p=0.0001$ and 0.02; **Fig. 4D, E**; representative images are provided in **Fig. S4**). No effect of CHLOR was instead detected on Fos expression in the striatum (treatment and genotype x treatment interaction: $F_{2,136} = 2.85$ and 0.78, NS; **Fig. 4F**).

Structural plasticity has been reported to be impaired in FXS patients, a phenotype also observed in *Fmr1*-KO mice which exhibit dysfunctional activity-dependent spine plasticity, morphology and maturation across hippocampal and cortical brain regions [59-62]. As expected, we confirmed this phenotype in our vehicle-treated *Fmr1*-KO mice. Compared to WT control littermates, hippocampal CA1 pyramidal neurons of *Fmr1*-KO mice showed increased spine density as well as an overabundance of immature dendritic spines (**Fig. 4G-I**). Interestingly, chronic administration of CHLOR at the dose of 5 mg/kg for 10 days corrected

this abnormal *Fmr1*-KO plasticity phenotype, without effects in WT mice (spine density: genotype x treatment interaction, $F_{1,12}$ = 18.89, p = 0.001; percent number of immature spines: interaction genotype x treatment, $F_{1,12}$ = 219.72, p < 0.0001; **Fig. 4H, I**).

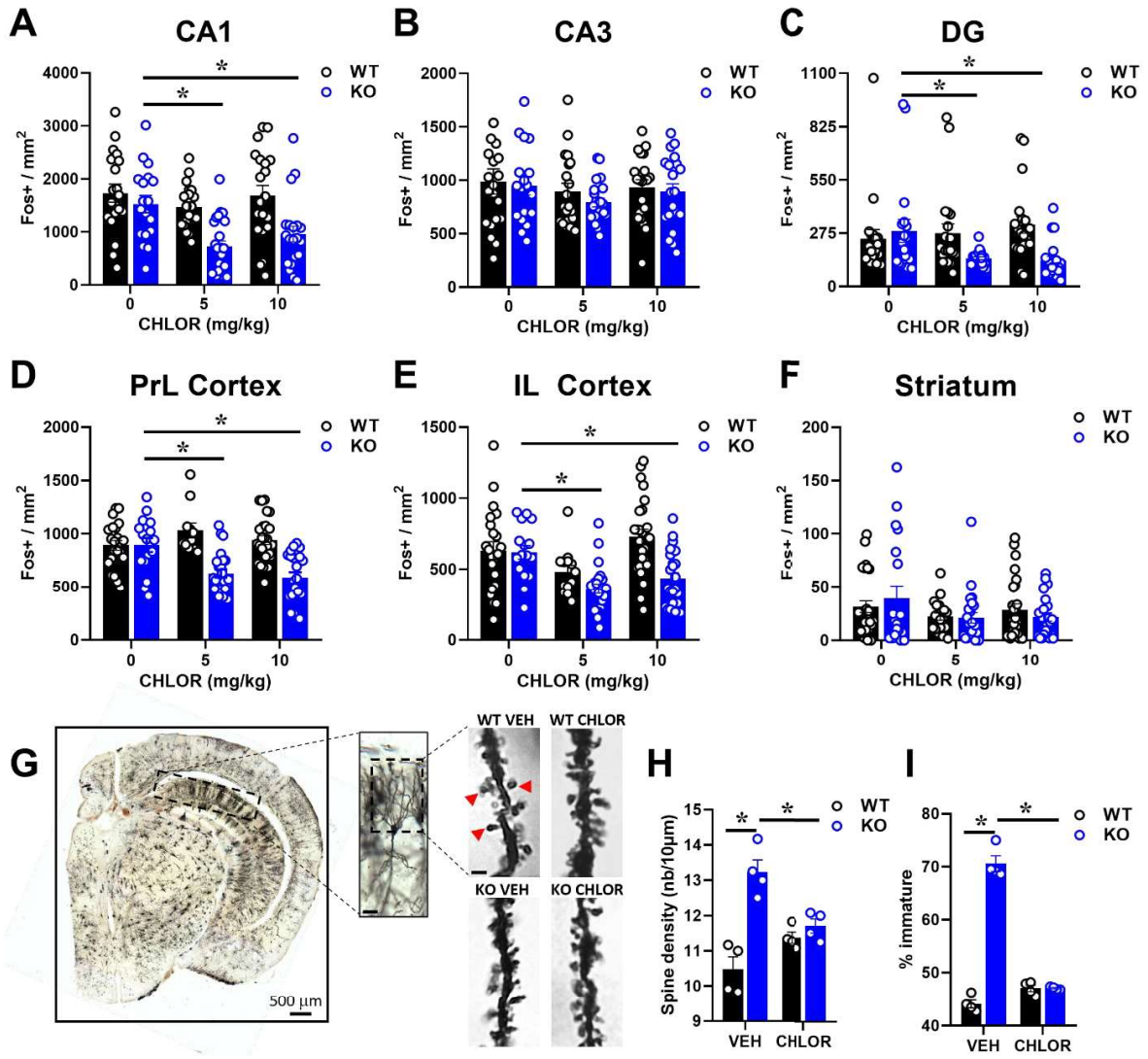

Fig. 4: Effects of chlorzoxazone on neuronal activity and structural plasticity of *Fmr1*-KO mice. Two different batches of behaviorally-naïve *Fmr1*-KO and WT mice were employed for Fos analysis (A to F) and the evaluation of CA1 dendritic spines (G-I). Neuronal activation following a single i.p. administration of CHLOR (either at 5 or 10 mg/kg) was evaluated through the quantification of Fos positive cells in hippocampal (A, B and C), cortical (D and E) and striatal (F) areas. DG = dentate gyrus; PrL = prelimbic; IL = infralimbic. Number of values per area: n=18-24 (CA1, CA3 and DG), 11-26 (PrL cortex), 13-16 (IL cortex), 20-28 (striatum), obtained from 5-6 mice per group. Spine density (H) and percentage of immature spines (I) were evaluated in *Fmr1*-KO mice and their WT littermates following 10 day-administration of CHLOR or vehicle (VEH). Dendritic spines (number of animals per condition = 4) were analyzed in the basal dendrites of CA1 pyramidal neurons (G, red arrows indicate mature spines). Representative images of Golgi-stained CA1 pyramidal neurons basal dendrites illustrating mature and immature spines in the different experimental groups (G). *p < 0.05. Data are mean ± SEM.

Chlorzoxazone rescues altered neuronal BKCa currents in *Fmr1*-KO mice

Because previous studies implicated BKCa channels in the neuropathogenesis of FXS [24, 25, 63], notably a reduced BKCa channel functional activity [22, 24], we first examined the status

of BKCa neuronal currents in vehicle-treated *Fmr1*-KO mice (**Fig. 5A-C**). We observed a clear deficit in BKCa currents recorded from hippocampal neurons of *Fmr1* mutants (genotype effect: $F_{1,40}$ = 8.42, p = 0.006; **Fig. 5C**). Next, to characterize the potential of CHLOR in upregulating BKCa neuronal currents, we again patch clamp-recorded hippocampal neurons in the presence of increasing concentrations of CHLOR. We found that CHLOR was able to normalize BKCa currents in a dose-dependent manner, with doses of 30 and 50 µM being efficacious in KOs without affecting BKCa currents of WT hippocampal neurons (genotype x treatment interaction: $F_{1,40}$ = 1.03, NS; treatment effect in KOs: $F_{3,20}$ = 3.42, p = 0.037; in WT: $F_{3,20}$ = 0.46, ns; **Fig. 5C**). The analysis of BKCa currents in cortical neurons revealed a highly similar pattern of effects. A marked deficit was observed in *Fmr1*-KO mice which was fully rescued by CHLOR at the concentration of 50 µM (genotype x treatment interaction: $F_{1,20}$ = 25.01, p < 0.0001; **Fig. 5D**).

We then evaluated the potential contribution of small-conductance calcium-dependent potassium (SKCa) channels to the therapeutic effects of CHLOR (**Fig. S5A-C**) since CHLOR is also known to stimulate these channels, albeit with a lower efficacy than BKCa [40, 64]. As expected [65], SK currents were also reduced in the hippocampus of *Fmr1* mutants, but this deficit was only partially rescued by CHLOR which was unable to restore SK currents to a level comparable to that of WT mice (genotype x treatment interaction: $F_{1,21}$ = 5.84, p = 0.025; **Fig. S5C**).

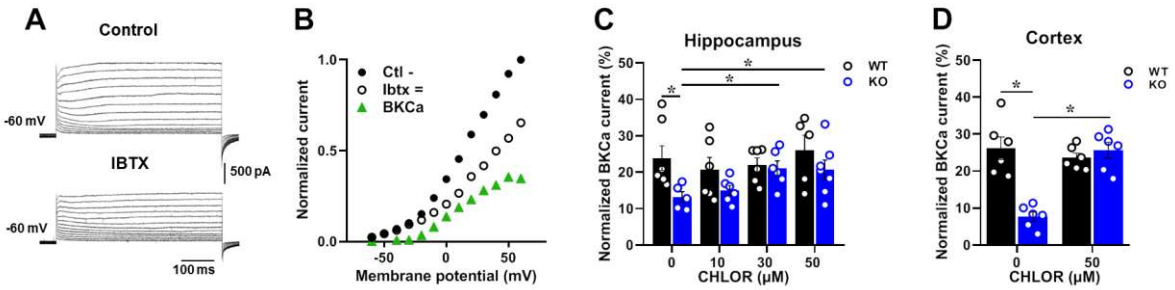

Fig. 5: Effects of chlorzoxazone (CHLOR) on hippocampal and cortical BKCa currents in *Fmr1*-KO mice and their WT littermates. Representative traces of currents evoked by steps of potentials from -60 mV to +60 mV in CA3 pyramidal neurons in the absence or presence of 200 nM iberiotoxin (IBTX, A). The current blocked by IBTX was considered as BKCa channel current (B). Bar graph and data plots of the normalized BKCa currents computed in the different experimental conditions tested in WT and KO mice in pyramidal neurons from CA3 (C) or cortical layer 5 (D). Neurons were obtained from 5-7 (C) or 6 (D) mice per condition. *p < 0.05. Data are mean ± SEM.

DISCUSSION

Our findings demonstrate the acute and chronic efficacy of the BKCa agonist CHLOR in rescuing major FXS-like behavioral abnormalities displayed by both the first and second generation *Fmr1*-KO mice models of FXS. Initially approved by the FDA for painful musculoskeletal conditions, CHLOR has been shown to activate BKCa channels [40], raising its potential for restoring the impaired BKCa functionality observed in FXS. We have indeed previously demonstrated the therapeutic effects of CHLOR on the acoustic abnormalities of *Fmr1*-KO mice, although these findings only concerned the acute effects of CHLOR and were restricted to the evaluation of cochlear functionality [33]. Using behavioral, cellular and electrophysiological approaches, we provide here novel evidence to support the repurposing value of CHLOR for treating neurodevelopmental disorders.

The therapeutic effects of CHLOR were demonstrated following both acute and chronic systemic administration, with higher efficacy than gaboxadol and metformin, two molecules previously proposed as repurposing treatments for FXS [10, 13, 66]. No significant aversive effects were observed following chronic (10 days) treatment with CHLOR, besides a slight weight loss of approximately 2% that was detected in only one of our studies. Overall, our data identified the 5 mg/kg dose of CHLOR as being the optimal treatment to induce the most robust behavioral rescue of the FXS-phenotype of *Fmr1*-KO mice across multiple behavioral readouts. Notably, this particular dose falls within the concentration range recommended in human posology (i.e., 250-500 mg/day for an adult subject [31]). Interestingly, acute positive effects of a lower dose (3.75 mg/kg corresponding to 75% of the initial 5 mg/kg dose) were observed on hyperactivity, thus suggesting that a reduction in the daily dose may be envisioned and still hold clinical potential. In particular, the possibility of using low or micro-doses [67] has marked clinical advantages with regard to the anticipated long-term administration regimen required to treat patients with neurodevelopmental disorders.

Importantly, the effects of CHLOR extended beyond behavioral rescue as revealed by the restoration of cerebral phenotypes of *Fmr1*-KO mice, concerning structural neuronal plasticity (i.e., correction of hippocampal dendritic spine abnormalities) and BKCa channel functioning (i.e., dose-dependent rescue of hippocampal BKCa currents). These brain effects of CHLOR could be linked, since BKCa channels modulate the excitatory-inhibitory (E/I) balance within neuronal circuits [68] and an E/I imbalance is implicated in the abnormal dendritic spine FXS phenotype [69]. Furthermore, the impaired interaction between FMRP

and BKCa channels due to *FMR1* deletion has been shown to enhance glutamate levels which in turn exacerbate neuronal excitability [26], two concomitant deleterious mechanisms that may be targeted by CHLOR via a normalization of BKCa currents, as observed in our *Fmr1*-KO mice. Alternatively, CHLOR might contribute to restoring the altered synaptic transmission induced by an impaired functional coupling of BKCa channels and NMDA receptors in basal dendrites of neuronal circuits [70].

No effects of CHLOR on macro-orchidism were detected, suggesting that central rather than peripheral BKCa channels may be preferentially targeted by this treatment, at least at the dose employed here. Our results may also suggest that BKCa channels are not involved in this FXS-like phenotype, although their testis expression is well documented [71]. The central efficacy of the systemic administration of CHLOR was confirmed by our Fos mapping results, indicating a selective effect of this compound in specific brain regions of *Fmr1*-KO mice.

Fos expression was clearly downregulated by a single administration of CHLOR in mutants and this effect was consistently observed in the hippocampal CA1 and dentate gyrus, and in the infralimbic and prelimbic subdivisions of the prefrontal cortex. This CHLOR-induced Fos reduction was not a general, non-specific, brain effect since it was not detected in the CA3 and in the striatum of *Fmr1*-KOs. This effect may be due to a direct activation of BKCa channels by CHLOR in these areas since BKCa inhibition by iberiotoxin is known to instead promote *c-fos* expression in brain slices [72]. The effect of CHLOR on Fos expression may also reflect the well-known, albeit complex, inhibitory impact of this BKCa channel opener on neuronal excitability [16]. Interestingly, while most drugs are known to increase cerebral Fos expression in preclinical models, a reduction in *c-fos* expression in cortical and hippocampal areas similar to the acute effect of CHLOR reported here was described in rats following anti-depressant administration (e.g., fluoxetine and vortioxetine, although chronically administered) [73]. Notably, the same antidepressants were able to promote the maturity of dendritic hippocampal spines [74], like CHLOR here, and to exert positive effects on synaptic plasticity [73, 74]. Furthermore, the effects of vortioxetine on Fos expression in WT animals were associated with a direct modulation of hippocampal *Fmr1* expression [75].

Unexpected however was the finding of a lack of difference in Fos expression between VEH-treated *Fmr1*-KOs mice and their WT littermates in the different brain regions analyzed. Such an absence of genotype effect is in partial disagreement with previous

reports showing increased Fos expression in the CA1 of *Fmr1*-KO mice [76] or reduced Fos labeling in their prefrontal cortex [77]. Another study described no difference in Fos expression between *Fmr1*-KO and WT littermates under basal conditions, but a Fos labeling increase in the amygdala following social exposure in *Fmr1* mutants [78]. These discrepancies among studies may be due to differences in *c-fos* assessment (e.g., quantification of mRNA versus Fos protein) or in the genetic background of the *Fmr1* mutation (e.g., the B6 versus the FVB strain), since the latter is known to affect several neurobehavioral phenotypes of *Fmr1*-KOs [43, 79, 80]. Furthermore, the basal conditions we used here differ from previous studies in *Fmr1*-KO mice because of the prior habituation to intraperitoneal injections and the related handling that all animals underwent in order to reduce non-specific Fos upregulation. This prior experience is known to exert effects on *c-fos* brain expression in mice [81] and could therefore contribute to explain the discrepancies with previous reports.

Overall, our results support the view that molecules activating BKCa channels may be a valid therapeutic strategy for treating FXS. This therapeutic avenue finds its roots in our previous studies showing that the acute administration of BMS-204352, another BKCa channel opener originally proposed to treat ischemic stroke, is able to eliminate several neurobehavioral abnormalities in *Fmr1*-KO mice [24, 25, 63], including dendritic alterations [24] and cortical hyper-excitability [25]. However, BMS-204352 not only failed to display clinical efficacy in stroke patients [82], but also exerted only acute [24, 25, 63] neurobehavioral effects in *Fmr1* mice, possibly because of its short half-life [24]. The comparison between CHLOR and two other treatments recently proposed for the treatment of FXS further strengthens the value of this molecule as a treatment for FXS. In the present experiments, we indeed could not detect any acute behavioral effect of metformin, in agreement with previous reports on the *Fmr1*-KO model [10], while the chronic effects of metformin were restricted to anxiety-like/repetitive behaviors. Regarding gaboxadol, slight acute behavioral effects were detected in line with previous results [13], but again, CHLOR was more powerful in inducing a full behavioral rescue of *Fmr1*-KO mice, especially following a chronic regimen.

In conclusion, our findings highlight the relevance of BKCa channels as a therapeutic target for neurodevelopmental disorders and identify CHLOR as a repurposed compound for treating FXS. Pharmacologically, CHLOR exhibit a mix affinity profile for both small

conductance calcium-activated potassium (SKCa) channels predominantly expressed in the nervous system and big conductance BKCa channels which are ubiquitously expressed throughout neuronal and non-neuronal cell types [17, 40, 64]. This raises the possibility that the ability of CHLOR to target multiple potassium channels, known to be dysregulated by the lack of FMRP in FXS [23, 26, 65], may magnify its overall therapeutic efficacy. Nonetheless, the present findings support a predominant action of CHLOR on BKCa channels, as demonstrated by its full rescue of BKCa currents in hippocampal and cortical neurons of *Fmr1*-KO mice and its partial reversal of SK channel functional deficits. Furthermore, CHLOR has recently been shown to exert beneficial neurobehavioral effects in preclinical models of neurodegenerative diseases [35, 36, 38, 39]. It is therefore possible that BKCa channels may represent a therapeutic target common to multiple disorders of the central nervous system [83]. This may be of particular importance for neurodevelopmental disorders, as alterations in BKCa channel functionality have been demonstrated in several of these pathologies [83], including FXS [26], but also ASD which are intertwined at the molecular [29, 84] and behavioral levels [85-88].

LIMITATIONS

One limitation of this study is the lack of experimental evidence concerning the molecular mechanisms by which CHLOR exerts its positive behavioral and brain effects in *Fmr1*-KO mice. Future studies should specifically address the impact of CHLOR on neuronal signaling pathways that are dysregulated in FXS, such as protein kinases (PKs), Wnt/β-catenin, ERK/MAPK, and mTOR [89-94], which are also a target of FMRP [29, 95-97]. Indeed, a complex interplay between these signaling pathways and BKCa channels has been suggested previously [98-104]. Given that these pathways are deregulated also in *Fmr1*-KO mice [92, 94, 105-109], it is plausible that CHLOR may exert its overall therapeutic effects through normalization of these pathways. Further studies should also investigate the precise modulating impact of CHLOR on neuronal calcium homeostasis (including its effects on Cav channels) that may in turn play a critical role in all or part of its effects, such as for instance its ability of reducing Fos expression in selected brain areas [110].

Finally, our results show that the efficacy of CHLOR is specific to *Fmr1-KO* mice as no behavioral or brain effects were observed in WT littermates, even with the highest dose of

CHLOR which also failed to alter BKCa or SKCa channel currents in WT neurons. A genotype-specific difference in neuronal potassium channel sensitivity to CHLOR may be hypothesized, the lack of FRMP in *Fmr1-KO* mice affecting the trafficking and subunit functionality of these channels [20], which may in turn affect their pharmacological responses [111]. Alternatively, it is possible that the absence of CHLOR effects on potassium currents in WT mice may be due to the unnecessity of increasing the functionality of potassium channels in non-pathological conditions, in line with previous studies showing no neuronal effects of other BKCa channel openers (e.g., BMS) in WT mice [25]. The latter hypothesis warrants future studies using single-cell recordings which will help disentangling the effects of CHLOR on neuronal functionality, taking into consideration the complex interactions occurring between BKCa channels and other ion channels at the neuronal level [112, 113].

AUTHOR CONTRIBUTIONS

Experimental activity, data collection and analysis: CF, MPL, VL, MMM, ST, BU, EC, SB, and SP. Study design: EL, BB and SP. Project supervision: SP. Manuscript writing: CF, BB and SP. All authors have reviewed and approved the manuscript.

COMPETING INTERESTS

VL, EL, and SP are inventors on the patent: “Methods of treatment and/or prevention of disorders and symptoms related to BKCa and/or SK channelopathies” (N°. EP20775228.8 and N°. US17760612) owned by CNRS. The remaining authors have nothing to disclose.

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REFERENCES

1. Pieretti M, Zhang FP, Fu YH, Warren ST, Oostra BA, Caskey CT, et al. Absence of expression of the FMR-1 gene in fragile X syndrome. Cell. 1991;66(4):817-22.
2. Santoro MR, Bray SM, Warren ST. Molecular mechanisms of fragile X syndrome: a twenty-year perspective. Annual review of pathology. 2012;7:219-45.
3. Greenough WT, Klintsova AY, Irwin SA, Galvez R, Bates KE, Weiler IJ. Synaptic regulation of protein synthesis and the fragile X protein. Proc Natl Acad Sci U S A. 2001;98(13):7101-6.
4. Tranfaglia MR. The psychiatric presentation of fragile x: evolution of the diagnosis and treatment of the psychiatric comorbidities of fragile X syndrome. Dev Neurosci. 2011;33(5):337-48.
5. Aishworiya R, Valica T, Hagerman R, Restrepo B. An Update on Psychopharmacological Treatment of Autism Spectrum Disorder. Neurotherapeutics. 2022;19(1):248-62. doi:10.1007/s13311-022-01183-1
6. Johnson D, Clark C, Hagerman R. Targeted Treatments for Fragile X Syndrome. Adv Neurobiol. 2023;30:225-53. doi:10.1007/978-3-031-21054-9_10
7. Protic D, Salcedo-Arellano MJ, Dy JB, Potter LA, Hagerman RJ. New Targeted Treatments for Fragile X Syndrome. Curr Pediatr Rev. 2019;15(4):251-8. doi:10.2174/1573396315666190625110748
8. Wang LW, Berry-Kravis E, Hagerman RJ. Fragile X: leading the way for targeted treatments in autism. Neurotherapeutics. 2010;7(3):264-74.
9. Wen TH, Binder DK, Ethell IM, Razak KA. The Perineuronal 'Safety' Net? Perineuronal Net Abnormalities in Neurological Disorders. Frontiers in molecular neuroscience. 2018;11:270. doi:10.3389/fnmol.2018.00270
10. Gantois I, Khoutorsky A, Popic J, Aguilar-Valles A, Freemantle E, Cao R, et al. Metformin ameliorates core deficits in a mouse model of fragile X syndrome. Nat Med. 2017;23(6):674-7. doi:10.1038/nm.4335
11. Gkogkas CG, Khoutorsky A, Cao R, Jafarnejad SM, Prager-Khoutorsky M, Giannakas N, et al. Pharmacogenetic inhibition of eIF4E-dependent Mmp9 mRNA translation reverses fragile X syndrome-like phenotypes. Cell Rep. 2014;9(5):1742-55. doi:10.1016/j.celrep.2014.10.064

12. Cogram P, Deacon RMJ, Warner-Schmidt JL, von Schimmelmann MJ, Abrahams BS, During MJ. Gaboxadol Normalizes Behavioral Abnormalities in a Mouse Model of Fragile X Syndrome. Front Behav Neurosci. 2019;13:141. doi:10.3389/fnbeh.2019.00141
13. Olmos-Serrano JL, Corbin JG, Burns MP. The GABA(A) receptor agonist THIP ameliorates specific behavioral deficits in the mouse model of fragile X syndrome. Developmental neuroscience. 2011;33(5):395-403.
14. Wafford KA, Ebert B. Gaboxadol--a new awakening in sleep. Curr Opin Pharmacol. 2006;6(1):30-6. doi:10.1016/j.coph.2005.10.004
15. Bartholini G. GABA receptor agonists: pharmacological spectrum and therapeutic actions. Med Res Rev. 1985;5(1):55-75. doi:10.1002/med.2610050103
16. N'Gouemo P. BKCa channel dysfunction in neurological diseases. Front Physiol. 2014;5:373. doi:10.3389/fphys.2014.00373
17. Kshatri AS, Gonzalez-Hernandez A, Giraldez T. Physiological Roles and Therapeutic Potential of Ca(2+) Activated Potassium Channels in the Nervous System. Frontiers in molecular neuroscience. 2018;11:258. doi:10.3389/fnmol.2018.00258
18. Ancaten-Gonzalez C, Segura I, Alvarado-Sanchez R, Chavez AE, Latorre R. Ca(2+)- and Voltage-Activated K(+) (BK) Channels in the Nervous System: One Gene, a Myriad of Physiological Functions. Int J Mol Sci. 2023;24(4). doi:10.3390/ijms24043407
19. Orfali R, Albanyan N. Ca(2+)-Sensitive Potassium Channels. Molecules. 2023;28(2). doi:10.3390/molecules28020885
20. Echeverria F, Gonzalez-Sanabria N, Alvarado-Sanchez R, Fernandez M, Castillo K, Latorre R. Large conductance voltage-and calcium-activated K(+) (BK) channel in health and disease. Front Pharmacol. 2024;15:1373507. doi:10.3389/fphar.2024.1373507
21. Deng PY, Klyachko VA. Genetic upregulation of BK channel activity normalizes multiple synaptic and circuit defects in a mouse model of fragile X syndrome. J Physiol. 2016;594(1):83-97. doi:10.1113/JP271031
22. Deng PY, Rotman Z, Blundon JA, Cho Y, Cui J, Cavalli V, et al. FMRP regulates neurotransmitter release and synaptic information transmission by modulating action potential duration via BK channels. Neuron. 2013;77(4):696-711. doi:10.1016/j.neuron.2012.12.018
23. Kshatri A, Cerrada A, Gimeno R, Bartolome-Martin D, Rojas P, Giraldez T. Differential regulation of BK channels by fragile X mental retardation protein. J Gen Physiol. 2020;152(6). doi:10.1085/jgp.201912502
24. Hebert B, Pietropaolo S, Meme S, Laudier B, Laugeray A, Doisne N, et al. Rescue of fragile X syndrome phenotypes in Fmr1 KO mice by a BKCa channel opener molecule. Orphanet journal of rare diseases. 2014;9:124.

25. Zhang Y, Bonnan A, Bony G, Ferezou I, Pietropaolo S, Ginger M, et al. Dendritic channelopathies contribute to neocortical and sensory hyperexcitability in Fmr1(-/y) mice. Nat Neurosci. 2014;17(12):1701-9. doi:10.1038/nn.3864
26. Myrick LK, Deng PY, Hashimoto H, Oh YM, Cho Y, Poidevin MJ, et al. Independent role for presynaptic FMRP revealed by an FMR1 missense mutation associated with intellectual disability and seizures. Proc Natl Acad Sci U S A. 2015;112(4):949-56. doi:10.1073/pnas.1423094112
27. Wang ZW. Regulation of synaptic transmission by presynaptic CaMKII and BK channels. Mol Neurobiol. 2008;38(2):153-66. doi:10.1007/s12035-008-8039-7
28. van Welie I, du Lac S. Bidirectional control of BK channel open probability by CAMKII and PKC in medial vestibular nucleus neurons. J Neurophysiol. 2011;105(4):1651-9. doi:10.1152/jn.00058.2011
29. Darnell JC, Van Driesche SJ, Zhang C, Hung KY, Mele A, Fraser CE, et al. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. Cell. 2011;146(2):247-61. doi:10.1016/j.cell.2011.06.013
30. Carreno-Munoz MI, Martins F, Medrano MC, Aloisi E, Pietropaolo S, Dechaud C, et al. Potential Involvement of Impaired BK(Ca) Channel Function in Sensory Defensiveness and Some Behavioral Disturbances Induced by Unfamiliar Environment in a Mouse Model of Fragile X Syndrome. Neuropsychopharmacology. 2018;43(3):492-502. doi:10.1038/npp.2017.149
31. Hohmann N, Blank A, Burhenne J, Suzuki Y, Mikus G, Haefeli WE. Simultaneous phenotyping of CYP2E1 and CYP3A using oral chlorzoxazone and midazolam microdoses. Br J Clin Pharmacol. 2019;85(10):2310-20. doi:10.1111/bcp.14040
32. Martindale W. the Extra Pharmacopoeia. 30th Edition: Amer Pharmaceutical Assn; 1993.
33. Ferraguto C, Bouleau Y, Peineau T, Dulon D, Pietropaolo S. Hyperacusis in the Adult Fmr1-KO Mouse Model of Fragile X Syndrome: The Therapeutic Relevance of Cochlear Alterations and BKCa Channels. Int J Mol Sci. 2023;24(14). doi:10.3390/ijms241411863
34. Deng L, Li H, Su X, Zhang Y, Xu H, Fan L, et al. Chlorzoxazone, a small molecule drug, augments immunosuppressive capacity of mesenchymal stem cells via modulation of FOXO3 phosphorylation. Cell Death Dis. 2020;11(3):158. doi:10.1038/s41419-020-2357-8
35. Bai Y, Ma X. Chlorzoxazone exhibits neuroprotection against Alzheimer's disease by attenuating neuroinflammation and neurodegeneration in vitro and in vivo. Int Immunopharmacol. 2020;88:106790. doi:10.1016/j.intimp.2020.106790
36. Egorova PA, Gavrilova AV, Bezprozvanny IB. Ataxic Symptoms in Huntington's Disease Transgenic Mouse Model Are Alleviated by Chlorzoxazone. Front Neurosci. 2020;14:279. doi:10.3389/fnins.2020.00279

37. Kasumu AW, Hougaard C, Rode F, Jacobsen TA, Sabatier JM, Eriksen BL, et al. Selective positive modulator of calcium-activated potassium channels exerts beneficial effects in a mouse model of spinocerebellar ataxia type 2. Chem Biol. 2012;19(10):1340-53. doi:10.1016/j.chembiol.2012.07.013
38. Marinina KS, Bezprozvanny IB, Egorova PA. A chlorzoxazone-folic acid combination improves cognitive affective decline in SCA2-58Q mice. Scientific reports. 2023;13(1):12588. doi:10.1038/s41598-023-39331-y
39. Egorova PA, Bezprozvanny IB. Electrophysiological Studies Support Utility of Positive Modulators of SK Channels for the Treatment of Spinocerebellar Ataxia Type 2. Cerebellum. 2022;21(5):742-9. doi:10.1007/s12311-021-01349-1
40. Liu YC, Lo YK, Wu SN. Stimulatory effects of chlorzoxazone, a centrally acting muscle relaxant, on large conductance calcium-activated potassium channels in pituitary GH3 cells. Brain Res. 2003;959(1):86-97. doi:10.1016/s0006-8993(02)03730-7
41. Jung YH, Hong SI, Ma SX, Hwang JY, Kim JS, Lee JH, et al. Strain differences in the chronic mild stress animal model of depression and anxiety in mice. Biomol Ther (Seoul). 2014;22(5):453-9. doi:10.4062/biomolther.2014.058
42. Oddi D, Crusio WE, D'Amato FR, Pietropaolo S. Monogenic mouse models of social dysfunction: Implications for autism. Behavioural brain research. 2013;251:75-84.
43. Pietropaolo S, Guilleminot A, Martin B, D'Amato FR, Crusio WE. Genetic-background modulation of core and variable autistic-like symptoms in FMR1 knock-out mice. PLoS One. 2011;6(2):e17073.
44. Pietropaolo S, Goubran MG, Joffre C, Aubert A, Lemaire-Mayo V, Crusio WE, et al. Dietary supplementation of omega-3 fatty acids rescues fragile X phenotypes in Fmr1-Ko mice. Psychoneuroendocrinology. 2014;49:119-29. doi:10.1016/j.psyneuen.2014.07.002
45. Pietropaolo S, Subashi E. Mouse models of Fragile X syndrome. In: Pietropaolo S, Sluyter F, Crusio WE, editors. Behavioral Genetics of the Mouse. Cambridge: Cambridge University Press; 2014. p. 146-63.
46. Mientjes EJ, Nieuwenhuizen I, Kirkpatrick L, Zu T, Hoogeveen-Westerveld M, Severijnen L, et al. The generation of a conditional Fmr1 knock out mouse model to study Fmrp function in vivo. Neurobiology of disease. 2006;21(3):549-55.
47. Yan QJ, Asafo-Adjei PK, Arnold HM, Brown RE, Bauchwitz RP. A phenotypic and molecular characterization of the fmr1-tm1Cgr fragile X mouse. Genes, brain, and behavior. 2004;3(6):337-59.
48. Gaudissard J, Ginger M, Premoli M, Memo M, Frick A, Pietropaolo S. Behavioral abnormalities in the Fmr1-KO2 mouse model of fragile X syndrome: The relevance of early life phases. Autism Res. 2017;10(10):1584-96. doi:10.1002/aur.1814

49. Gauducheau M, Lemaire-Mayo V, D'Amato FR, Oddi D, Crusio WE, Pietropaolo S. Age-specific autistic-like behaviors in heterozygous Fmr1-KO female mice. Autism Res. 2017;10(6):1067-78. doi:10.1002/aur.1743
50. Kat R, Arroyo-Araujo M, de Vries RBM, Koopmans MA, de Boer SF, Kas MJH. Translational validity and methodological underreporting in animal research: A systematic review and meta-analysis of the Fragile X syndrome (Fmr1 KO) rodent model. Neurosci Biobehav Rev. 2022;139:104722. doi:10.1016/j.neubiorev.2022.104722
51. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS biology. 2010;8(6):e1000412. doi:10.1371/journal.pbio.1000412
52. Oddi D, Subashi E, Middei S, Bellocchio L, Lemaire-Mayo V, Guzman M, et al. Early social enrichment rescues adult behavioral and brain abnormalities in a mouse model of fragile X syndrome. Neuropsychopharmacology. 2015;40(5):1113-22. doi:10.1038/npp.2014.291
53. Petroni V, Subashi E, Premoli M, Memo M, Lemaire V, Pietropaolo S. Long-term behavioral effects of prenatal stress in the Fmr1-knock-out mouse model for fragile X syndrome. Frontiers in cellular neuroscience. 2022;16:917183. doi:10.3389/fncel.2022.917183
54. Premoli M, Fyke W, Bellocchio L, Lemaire V, Wolley-Roberts M, Bontempi B, et al. Early Administration of the Phytocannabinoid Cannabidivarin Prevents the Neurobehavioral Abnormalities Associated with the Fmr1-KO Mouse Model of Fragile X Syndrome. Cells. 2023;12(15). doi:10.3390/cells12151927
55. De Arce MA, Kearns A. The fragile X syndrome: the patients and their chromosomes. J Med Genet. 1984;21(2):84-91. doi:10.1136/jmg.21.2.84
56. Hagerman RJ, Amiri K, Cronister A. Fragile X checklist. Am J Med Genet. 1991;38(2-3):283-7. doi:10.1002/ajmg.1320380223
57. Herrera DG, Robertson HA. Activation of c-fos in the brain. Prog Neurobiol. 1996;50(2-3):83-107. doi:10.1016/s0301-0082(96)00021-4
58. Zhang J, Zhang D, McQuade JS, Behbehani M, Tsien JZ, Xu M. c-fos regulates neuronal excitability and survival. Nat Genet. 2002;30(4):416-20. doi:10.1038/ng859
59. Hagerman R, Au J, Hagerman P. FMR1 premutation and full mutation molecular mechanisms related to autism. Journal of neurodevelopmental disorders. 2011;3(3):211-24. doi:10.1007/s11689-011-9084-5
60. Booker SA, Domanski APF, Dando OR, Jackson AD, Isaac JTR, Hardingham GE, et al. Altered dendritic spine function and integration in a mouse model of fragile X syndrome. Nature communications. 2019;10(1):4813. doi:10.1038/s41467-019-11891-6

61. Dictenberg JB, Swanger SA, Antar LN, Singer RH, Bassell GJ. A direct role for FMRP in activity-dependent dendritic mRNA transport links filopodial-spine morphogenesis to fragile X syndrome. Dev Cell. 2008;14(6):926-39. doi:10.1016/j.devcel.2008.04.003
62. Irwin SA, Galvez R, Greenough WT. Dendritic spine structural anomalies in fragile-X mental retardation syndrome. Cereb Cortex. 2000;10(10):1038-44. doi:10.1093/cercor/10.10.1038
63. Carreno-Munoz MI, Martins F, Medrano MC, Aloisi E, Pietropaolo S, Dechaud C, et al. Potential Involvement of Impaired BKCa Channel Function in Sensory Defensiveness and Some Behavioral Disturbances Induced by Unfamiliar Environment in a Mouse Model of Fragile X Syndrome. Neuropsychopharmacology. 2018;43(3):492-502. doi:10.1038/npp.2017.149
64. Cao Y, Dreixler JC, Roizen JD, Roberts MT, Houamed KM. Modulation of recombinant small-conductance Ca(2+)-activated K(+) channels by the muscle relaxant chlorzoxazone and structurally related compounds. J Pharmacol Exp Ther. 2001;296(3):683-9.
65. Deng PY, Carlin D, Oh YM, Myrick LK, Warren ST, Cavalli V, et al. Voltage-Independent SK-Channel Dysfunction Causes Neuronal Hyperexcitability in the Hippocampus of Fmr1 Knock-Out Mice. J Neurosci. 2019;39(1):28-43. doi:10.1523/JNEUROSCI.1593-18.2018
66. Biag HMB, Potter LA, Wilkins V, Afzal S, Rosvall A, Salcedo-Arellano MJ, et al. Metformin treatment in young children with fragile X syndrome. Mol Genet Genomic Med. 2019;7(11):e956. doi:10.1002/mgg3.956
67. Polito V, Liknaitzky P. The emerging science of microdosing: A systematic review of research on low dose psychedelics (1955-2021) and recommendations for the field. Neurosci Biobehav Rev. 2022;139:104706. doi:10.1016/j.neubiorev.2022.104706
68. Griguoli M, Sgritta M, Cherubini E. Presynaptic BK channels control transmitter release: physiological relevance and potential therapeutic implications. The Journal of physiology. 2016;594(13):3489-500. doi:10.1113/JP271841
69. Portera-Cailliau C. Which comes first in fragile X syndrome, dendritic spine dysgenesis or defects in circuit plasticity? Neuroscientist. 2012;18(1):28-44. doi:10.1177/1073858410395322
70. Gomez R, Maglio LE, Gonzalez-Hernandez AJ, Rivero-Perez B, Bartolome-Martin D, Giraldez T. NMDA receptor-BK channel coupling regulates synaptic plasticity in the barrel cortex. Proc Natl Acad Sci U S A. 2021;118(35). doi:10.1073/pnas.2107026118
71. Matzkin ME, Lauf S, Spinnler K, Rossi SP, Kohn FM, Kunz L, et al. The Ca2+-activated, large conductance K+-channel (BKCa) is a player in the LH/hCG signaling cascade in testicular Leydig cells. Mol Cell Endocrinol. 2013;367(1-2):41-9. doi:10.1016/j.mce.2012.12.015
72. Saria A, Fischer HS, Humpel C, Pfattner A, Schatz DS, Schuligoi R. Margatoxin and iberiotoxin, two selective potassium channel inhibitors, induce c-fos like protein and mRNA in rat organotypic dorsal striatal slices. Amino Acids. 2000;19(1):23-31. doi:10.1007/s007260070030

73. Waller JA, Tamm JA, Abdourahman A, Pehrson AL, Li Y, Cajina M, et al. Chronic vortioxetine treatment in rodents modulates gene expression of neurodevelopmental and plasticity markers. Eur Neuropsychopharmacol. 2017;27(2):192-203. doi:10.1016/j.euroneuro.2016.11.014
74. Waller JA, Nygaard SH, Li Y, du Jardin KG, Tamm JA, Abdourahman A, et al. Neuroplasticity pathways and protein-interaction networks are modulated by vortioxetine in rodents. BMC Neurosci. 2017;18(1):56. doi:10.1186/s12868-017-0376-x
75. Li Y, Abdourahman A, Tamm JA, Pehrson AL, Sanchez C, Gulinello M. Reversal of age-associated cognitive deficits is accompanied by increased plasticity-related gene expression after chronic antidepressant administration in middle-aged mice. Pharmacol Biochem Behav. 2015;135:70-82. doi:10.1016/j.pbb.2015.05.013
76. Lauterborn JC. Stress induced changes in cortical and hypothalamic c-fos expression are altered in fragile X mutant mice. Brain Res Mol Brain Res. 2004;131(1-2):101-9. doi:10.1016/j.molbrainres.2004.08.014
77. Krueger DD, Osterweil EK, Chen SP, Tye LD, Bear MF. Cognitive dysfunction and prefrontal synaptic abnormalities in a mouse model of fragile X syndrome. Proc Natl Acad Sci U S A. 2011;108(6):2587-92. doi:10.1073/pnas.1013855108
78. Rogers TD, Anacker AMJ, Kerr TM, Forsberg CG, Wang J, Zhang B, et al. Effects of a social stimulus on gene expression in a mouse model of fragile X syndrome. Molecular autism. 2017;8:30. doi:10.1186/s13229-017-0148-6
79. Errijgers V, Fransen E, D'Hooge R, De Deyn PP, Kooy RF. Effect of genetic background on acoustic startle response in fragile X knockout mice. Genet Res. 2008;90(4):341-5.
80. Spencer CM, Alekseyenko O, Hamilton SM, Thomas AM, Serysheva E, Yuva-Paylor LA, et al. Modifying behavioral phenotypes in Fmr1KO mice: genetic background differences reveal autistic-like responses. Autism Res. 2011;4(1):40-56.
81. Asanuma M, Ogawa N. Pitfalls in assessment of c-fos mRNA expression in the brain: effects of animal handling. Rev Neurosci. 1994;5(2):171-8. doi:10.1515/revneuro.1994.5.2.171
82. Jensen BS. BMS-204352: a potassium channel opener developed for the treatment of stroke. CNS Drug Rev. 2002;8(4):353-60.
83. Bailey CS, Moldenhauer HJ, Park SM, Keros S, Meredith AL. KCNMA1-linked channelopathy. J Gen Physiol. 2019;151(10):1173-89. doi:10.1085/jgp.201912457
84. Parikshak NN, Luo R, Zhang A, Won H, Lowe JK, Chandran V, et al. Integrative functional genomic analyses implicate specific molecular pathways and circuits in autism. Cell. 2013;155(5):1008-21. doi:10.1016/j.cell.2013.10.031

85. Bailey DB, Jr., Hatton DD, Skinner M, Mesibov G. Autistic behavior, FMR1 protein, and developmental trajectories in young males with fragile X syndrome. J Autism Dev Disord. 2001;31(2):165-74.
86. Bailey DB, Jr., Mesibov GB, Hatton DD, Clark RD, Roberts JE, Mayhew L. Autistic behavior in young boys with fragile X syndrome. J Autism Dev Disord. 1998;28(6):499-508.
87. Brock M, Hatton D. Distinguishing features of autism in boys with fragile X syndrome. J Intellect Disabil Res. 2010.
88. Rogers SJ, Wehner DE, Hagerman R. The behavioral phenotype in fragile X: symptoms of autism in very young children with fragile X syndrome, idiopathic autism, and other developmental disorders. J Dev Behav Pediatr. 2001;22(6):409-17.
89. Gross C, Bassell GJ. Excess protein synthesis in FXS patient lymphoblastoid cells can be rescued with a p110beta-selective inhibitor. Mol Med. 2012;18(1):336-45. doi:10.2119/molmed.2011.00363
90. Gross C, Nakamoto M, Yao X, Chan CB, Yim SY, Ye K, et al. Excess phosphoinositide 3-kinase subunit synthesis and activity as a novel therapeutic target in fragile X syndrome. J Neurosci. 2010;30(32):10624-38. doi:10.1523/JNEUROSCI.0402-10.2010
91. Hoeffer CA, Sanchez E, Hagerman RJ, Mu Y, Nguyen DV, Wong H, et al. Altered mTOR signaling and enhanced CYFIP2 expression levels in subjects with fragile X syndrome. Genes Brain Behav. 2012;11(3):332-41. doi:10.1111/j.1601-183X.2012.00768.x
92. Sharma A, Hoeffer CA, Takayasu Y, Miyawaki T, McBride SM, Klann E, et al. Dysregulation of mTOR signaling in fragile X syndrome. J Neurosci. 2010;30(2):694-702. doi:10.1523/JNEUROSCI.3696-09.2010
93. Song C, Broadie K. Dysregulation of BMP, Wnt, and Insulin Signaling in Fragile X Syndrome. Front Cell Dev Biol. 2022;10:934662. doi:10.3389/fcell.2022.934662
94. Wang X, Snape M, Klann E, Stone JG, Singh A, Petersen RB, et al. Activation of the extracellular signal-regulated kinase pathway contributes to the behavioral deficit of fragile x-syndrome. J Neurochem. 2012;121(4):672-9. doi:10.1111/j.1471-4159.2012.07722.x
95. Casingal CR, Kikkawa T, Inada H, Sasaki Y, Osumi N. Identification of FMRP target mRNAs in the developmental brain: FMRP might coordinate Ras/MAPK, Wnt/beta-catenin, and mTOR signaling during corticogenesis. Mol Brain. 2020;13(1):167. doi:10.1186/s13041-020-00706-1
96. Pedini G, Buccarelli M, Bianchi F, Pacini L, Cencelli G, D'Alessandris QG, et al. FMRP modulates the Wnt signalling pathway in glioblastoma. Cell Death Dis. 2022;13(8):719. doi:10.1038/s41419-022-05019-w

97. Sears JC, Choi WJ, Broadie K. Fragile X Mental Retardation Protein positively regulates PKA anchor Rugose and PKA activity to control actin assembly in learning/memory circuitry. Neurobiol Dis. 2019;127:53-64. doi:10.1016/j.nbd.2019.02.004

98. Bollinger WL, Sial N, Dawson-Scully K. BK channels and a cGMP-dependent protein kinase (PKG) function through independent mechanisms to regulate the tolerance of synaptic transmission to acute oxidative stress at the Drosophila larval neuromuscular junction. J Neurogenet. 2018;32(3):246-55. doi:10.1080/01677063.2018.1500571

99. Mitrokhin MV, Kalsin V, Kamkina O, Babkina I, Zotov A, Troitskiy VA, et al. Participation of PKG and PKA-related pathways in the IFN-gamma induced modulation of the BK(Ca) channel activity in human cardiac fibroblasts. J Pharmacol Sci. 2019;141(1):25-31. doi:10.1016/j.jphs.2019.08.006

100. Sansom SC, Ma R, Carmines PK, Hall DA. Regulation of Ca(2+)-activated K(+) channels by multifunctional Ca(2+)/calmodulin-dependent protein kinase. Am J Physiol Renal Physiol. 2000;279(2):F283-8. doi:10.1152/ajprenal.2000.279.2.F283

101. Schubert R, Nelson MT. Protein kinases: tuners of the BKCa channel in smooth muscle. Trends Pharmacol Sci. 2001;22(10):505-12. doi:10.1016/s0165-6147(00)01775-2

102. Wang F, Chen Q, Huang G, Guo X, Li N, Li Y, et al. BKCa participates in E2 inducing endometrial adenocarcinoma by activating MEK/ERK pathway. BMC Cancer. 2018;18(1):1128. doi:10.1186/s12885-018-5027-9

103. Wang Y, Tao J, Wang M, Yang L, Ning F, Xin H, et al. Mechanism of Regulation of Big-Conductance Ca(2+)-Activated K(+) Channels by mTOR Complex 2 in Podocytes. Front Physiol. 2019;10:167. doi:10.3389/fphys.2019.00167

104. Zhou X, Wulfsen I, Korth M, McClafferty H, Lukowski R, Shipston MJ, et al. Palmitoylation and membrane association of the stress axis regulated insert (STREX) controls BK channel regulation by protein kinase C. J Biol Chem. 2012;287(38):32161-71. doi:10.1074/jbc.M112.386359

105. Chen LY, Rex CS, Babayan AH, Kramar EA, Lynch G, Gall CM, et al. Physiological activation of synaptic Rac>PAK (p-21 activated kinase) signaling is defective in a mouse model of fragile X syndrome. J Neurosci. 2010;30(33):10977-84. doi:10.1523/JNEUROSCI.1077-10.2010

106. Guo W, Ceolin L, Collins KA, Perroy J, Huber KM. Elevated CaMKIIalpha and Hyperphosphorylation of Homer Mediate Circuit Dysfunction in a Fragile X Syndrome Mouse Model. Cell Rep. 2015;13(10):2297-311. doi:10.1016/j.celrep.2015.11.013

107. Jiang A, Wang L, Lu JYD, Freeman A, Campbell C, Su P, et al. Sex Differences in Dopamine Receptor Signaling in Fmr1 Knockout Mice: A Pilot Study. Brain Sci. 2021;11(11). doi:10.3390/brainsci11111398

108. Mok-Lin E, Ascano M, Jr., Serganov A, Rosenwaks Z, Tuschl T, Williams Z. Premature recruitment of oocyte pool and increased mTOR activity in Fmr1 knockout mice and reversal of phenotype with rapamycin. Sci Rep. 2018;8(1):588. doi:10.1038/s41598-017-18598-y

109. Sawicka K, Pyronneau A, Chao M, Bennett MV, Zukin RS. Elevated ERK/p90 ribosomal S6 kinase activity underlies audiogenic seizure susceptibility in fragile X mice. Proc Natl Acad Sci U S A. 2016;113(41):E6290-E7. doi:10.1073/pnas.1610812113

110. Morgan JI, Curran T. Calcium as a modulator of the immediate-early gene cascade in neurons. Cell Calcium. 1988;9(5-6):303-11. doi:10.1016/0143-4160(88)90011-5

111. Gonzalez-Perez V, Lingle CJ. Regulation of BK Channels by Beta and Gamma Subunits. Annu Rev Physiol. 2019;81:113-37. doi:10.1146/annurev-physiol-022516-034038

112. Blomer LA, Giacalone E, Abbas F, Filipis L, Tegolo D, Migliore M, et al. Kinetics and functional consequences of BK channels activation by N-type Ca(2+) channels in the dendrite of mouse neocortical layer-5 pyramidal neurons. Frontiers in cellular neuroscience. 2024;18:1353895. doi:10.3389/fncel.2024.1353895

113. Filipis L, Blomer LA, Montnach J, Loussouarn G, De Waard M, Canepari M. Nav1.2 and BK channel interaction shapes the action potential in the axon initial segment. The Journal of physiology. 2023;601(10):1957-79. doi:10.1113/JP283801