The novel potent GSK3 inhibitor AF3581 reverts Fragile X Syndrome phenotype

Pier Francesca Porceddu[1], Mariasole Ciampoli[1], Elisa Romeo[1], Beatrice Garrone[2], Lucia Durando[2], Claudio Milanese[2], Francesco Paolo Di Giorgio[2] and Angelo Reggiani[1]*

[1] D3 Validation Research Line, Istituto Italiano di Tecnologia, Via Morego 30, (16163) Genova, Italy

[2] Angelini Pharma S.p.A. Viale Amelia 70, 00181 Rome, Italy

- *Pier Francesca Porceddu[1]*,
- *Mariasole Ciampoli[1]*,
- *Elisa Romeo[1]*,
- *Beatrice Garrone[2]*,
- *Lucia Durando[2]*,
- *Claudio Milanese[2]*,
- *Francesco Paolo Di Giorgio[2]*,
- *Angelo Reggiani[1]*.*

Corresponding Author:

*Angelo Reggiani**

D3 Validation Research Line, Istituto Italiano di Tecnologia,

Via Morego 30, (16163) Genova, Italy

e-mail: angelo.reggiani@iit.it

Abstract

Glycogen synthase kinase 3 (GSK3) is a kinase mediating phosphorylation on serine and threonine amino acid residues of several target molecules. The enzyme is involved in the regulation of many cellular processes and aberrant activity of GSK3 has been linked to several disease conditions such as Fragile X Syndrome (FXS). Recent evidences demonstrating an increased activity of GSK3 in murine models of FXS, suggest that dysregulation/hyperactivation of the GSK3 path should contribute to FXS development. A likely possibility could be that in FXS there is a functional impairment of the upstream inhibitory input over GSK3 thus making overactive the kinase. Since GSK3 signaling is a central regulatory node for critical neurodevelopmental pathways, understanding the contribution of GSK3 dysregulation to FXS, may provide novel targets for therapeutic interventions for this disease. In this study we used AF3581, a potent GSK3 inhibitor that we recently discovered, in an *in vivo* FXS mouse model to elucidate the crucial role of GSK3 in specific behavioral patterns (locomotor activity, sensorimotor gating and social behavior) associated with this disease. All the behavioral alterations manifested by *Fmr1* knockout mice were reverted after a chronic treatment with our GSK3 inhibitor, confirming the importance of this pathway as a therapeutic target.

Introduction

Fragile X Syndrome (FXS) is one of the most common hereditary forms of mental retardation with a high incidence of phenotypic features typical of Autism Spectrum Disorders (ASDs). FXS is caused by a single genetic defect which causes the loss of expression of the fragile X mental retardation 1 (*Fmr1*) gene. In detail, a trinucleotide repeat expansion in the X-linked *Fmr1* gene (over 200–230 CGG repeats in the 5′ UTR) silences this gene transcription and abolishes the expression of Fragile X Mental Retardation Protein (FMRP) [1]. FMRP is a selective RNA-binding protein involved in a variety of developmental processes, such as neurogenesis, synaptic plasticity and dendritic development through interaction with a variety of signaling pathways [2; 3]. In particular, FMRP is known to play a critical role in adult hippocampal neurogenesis and regulates adult neural stem cell fate by modulating the translation of Glycogen-synthase kinase 3β (GSK3β) [4].

GSK3 is a serine/threonine kinase primarily located in the cell cytosol, although additional prevalent locations are mitochondria and nucleus [5]. Initially identified as a key regulator of insulin-dependent glycogen synthesis, GSK3 is at the crossroad of many pathways and for this reason has been implicated in diverse biological actions [6]. In mammals GSK3 is encoded by two different genes, GSK3α and GSK3β. These paralogs share nearly 98% homology in their kinase domain and often perform similar functions or may have redundant activity for substrates such as β-catenin [7].

GSK3 acts by phosphorylating downstream substrates, this action results in the inhibition of the target protein [8]. In particular, GSK3 transfers phosphate groups from ATP to Ser/Thr of specific substrates, regulating a plethora of physiological functions and cellular events, such as insulin pathway, Wnt signaling pathway, cell division, survival and death, neuronal development, and transcription [9; 10; 11]. GSK3 activity is in turn negatively regulated via phosphorylation at ser21 or ser9 of GSK3α and GSK3β, respectively [12].

GSK3 is widespread in many tissues with maximum levels found in the central nervous system (CNS), suggesting a fundamental role in neuronal signaling pathways. In particular, the two GSK3 isozymes have a distinct distribution in the central nervous system: GSK3α is more abundant in hippocampus, cerebral cortex, striatum, and cerebellum, while GSK3β is expressed in nearly all brain regions [7]. A significant functional

difference between the isoforms is clearly observed in embryonic development, but it is more complex to distinguish the roles played by the α and β isozymes in adult neurons [13].

In general, GSK3 modulates neuronal function in terms of neurogenesis, synaptic plasticity, neuronal structure, and neuronal survival and death [14]. In resting conditions GSK3 is functionally inhibited by upstream mechanisms while an aberrant elevated activity has been reported in neurological or neurodevelopmental diseases including FXS [15; 16; 17]. A likely possibility could be that in FXS there is a functional impairment of the upstream inhibitory input over GSK3 thus making overactive this kinase.

Recent evidences demonstrating an increased activity of both GSK3α and β isoforms in murine models of FXS [17; 18] suggest that dysregulation/hyperactivation of the GSK3 path should contribute to FXS pathogenesis. Treatment with GSK-3 inhibitors has been shown to improve FXS behavioral symptoms. Indeed, Franklin and colleagues first reported in 2014 that GSK3β inhibition reverses defective long-term potentiation and cognition in *Fmr1* knockout (KO) mice [18]. Additionally, in a very recent paper by McCamphill and colleagues describe the effects of GSK3α isoform specific inhibition in correcting different phenotypes of *Fmr1* KO mice [19].

Since GSK3 signaling is a central regulatory node for critical neurodevelopmental pathways, the understanding of how GSK3 aberrant activity is related to FXS development may provide a novel therapeutic clue for FXS and related ASDs.

On these bases, we recently started a screening program to develop GSK3 inhibitors with good *in vivo* efficacy and safety profile associated with high brain exposure. This effort led to the identification of AF3581, a potent GSK3 inhibitor [20; 21]. AF3581, in addition to a potent GSK3 inhibitory activity, shows a good kinase selectivity profile [19]. Finally, a promising pharmacokinetics (PK) profile was also observed and at the dose of 10 mg/kg intraperitoneally (i.p.) administrated we found high exposure both in plasma and brain [21].

In previous studies, we demonstrated that AF3581 has an excellent efficacy profile as mood-stabilizer without sedative effects in mouse models of mood disorders [22; 23]. Since, similarly to FXS, the pathophysiology of mood disorders has been linked to GSK3 aberrant activity, we thought that AF3581 would be a good tool to elucidate the role of GSK3 in FXS models. We chose to perform experiments in *Fmr1* KO mice (FVB/NJ background), a murine model, which is believed to be the most relevant preclinical model for FXS since it carries the deletion of *Fmr1* gene (the same gene silenced in FXS patients) and displays the main FXS-related behavioral phenotypes [24; 25; 26].

In order to establish whether GSK3 dysregulation occurred in this murine model, we first assessed GSK3 activity in *Fmr1* KO mice by determining the levels of phosphorylated βcatenin (p-βcatenin) [27; 28]. We next tested the *in vivo* efficacy of AF3581, compared with trofinetide, a new drug reported to ameliorate FXS symptoms in Phase II clinical trials [29], with a battery of behavioral tests (locomotor activity, sensorimotor gating and social behavior) in the FXS murine model.

Results

Increased expression of p-βcatenin reveals a GSK3 hyperactivity in *Fmr1* KO mice.

To analyze the activity of GSK3 in our FXS murine model, we measured the levels of p-βcatenin, which represent GSK3 principal target, in different tissues. As shown in Fig. 1, higher levels of p-βcatenin were observed in liver of *Fmr1* KO mice as respect to wild type (WT) animals, confirming an elevated GSK3 activity. We obtained similar results when analyzing lung tissue, whereas we were not able to reproduce data in brain (not shown). This could be explained with mouse strain issues. Indeed, despite GSK3 hyperactivity has been well characterized in different brain areas of *Fmr1* KO C57BL6 mice [17], the different levels of brain GSK3 activity between WT and *Fmr1* KO genotypes has been more complicated to be detected in mice with FVB background [30].

To demonstrate the GSK3 overactivation (and the consequent dysregulation of βcatenin signaling) also in brain tissues of our FXS murine model, we evaluated the p-βcatenin levels in primary neuronal cultures derived from WT and *Fmr1* KO mice respectively. Since phosphorylated βcatenin are known to be eliminated via ubiquitination and subsequent degradation by proteasome, to better evaluate the rate of βcatenin phosphorylation without this bias, we introduced a blocker of the proteolytic activity of the 26S proteasome, MG132 (carbobenzoxy-Leu-Leu-leucinal). As shown in Fig. 2, despite the basal levels of p-βcatenin were similar in WT and *Fmr1* KO mice-derived neurons, in presence of the MG132 proteasome inhibitor an amplified p-βcatenin accumulation was observed in *Fmr1* KO neurons as respect to WT. In presence of AF3581, the p-βcatenin accumulation was totally abolished, confirming that the diverse rate of βcatenin phosphorylation observed was due to different extent of GSK3 activity between WT and *Fmr1* KO mice-derived neurons.

The GSK3 inhibition abolished hyperactive phenotype in *Fmr1* KO mice.

The effect of AF3581 on hyperactivity of *Fmr1* KO mice was measured in the open field test. Our results confirmed an increased locomotor activity in our *Fmr1* deficiency murine model respect to WT, while a significant reduction of this hyperactive behavior was observed in *Fmr1* KO mice treated chronically with AF3581. However, to compare the efficacy of our compound with a reference compound, we tested the effect of chronic administrations of trofinetide in *Fmr1* KO mice. As shown in Fig. 3, a significant reduction of hyperactivity was seen in *Fmr1* KO mice treated with AF3581 (Fig. 3A), but not with trofinetide (Fig. 3B) with respect to *Fmr1* KO vehicle group.

Finally, the normal locomotor activity observed in WT mice treated with AF3581 suggested that the effect of our compound on hyperactivity is not mediated by sedation but is induced by the long-term molecular modification caused by the prolonged inhibition of GSK3. To corroborate this hypothesis, we performed the open field test 1 hour after a single dose of AF3581. In this case, the GSK3 inhibitor was not able to revert the hyperactive behavior observed in *Fmr1* KO mice (Fig. 3C), confirming the absence of sedative effect.

AF3581 restored normal sensorimotor functions in *Fmr1* KO mice.

FXS patients are hypersensitive to many different sensory stimuli [31], here we explored the sensorimotor deficits using the acoustic startle response test. This test is considered very stressful and for this reason it was the last to be performed. Usually, there is a sensorimotor response in consequence to repeated elevated acoustic stimuli; the absence or an extreme response to the stimulus could indicate the presence of sensorimotor deficits. In the acoustic startle response test, we measured the mouse response first in absence ("no stimulus") and then in presence ("startle") of an elevated acoustic stimulus (120 decibel (dB) of amplitude and 40 milliseconds of duration).

As shown in Fig. 4, both WT and *Fmr1* KO mice treated with AF3581 or vehicle, do not display response in presence of no stimulus. In presence of the elevated acoustic stimulus (startle), on the contrary, a variable response was seen. Confirming previous studies, *Fmr1* KO vehicle group showed higher startle reactivity to the 120 dB stimulus compared with WT. This *Fmr1*KO hyper-response was completely prevented by AF3581 treatment (Fig. 4A). The high reactivity of *Fmr1* KO mice to the 120 dB stimulus was interpreted as a sensorimotor deficit that the chronic treatment with AF3581 was able to revert. Interestingly some inhibition

of startle response was seen also in WT mice. The effect was only a trend but not significant. On the contrary, trofinetide did not completely abolish the abnormal response in *Fmr1* KO mice and consequently was unable to completely revert sensorimotor deficits typical of FXS (Fig. 4B).

Social deficits of *Fmr1* KO mice were reverted by AF3581, increasing friendly social behavior and reducing the aggressiveness.

The social habituation/dishabituation test evaluates social interactions and recognition of an unfamiliar mouse. After 1 hour of isolation in the testing cage, the test mouse was exposed to an unknown mouse (stimulus 1) for a test time of 1 minute (min). This sequence was repeated for 4 trials with 3 min inter-trial intervals introducing the same stimulus mouse in all 4 trials. Finally, in the fifth dishabituation trial, a new unfamiliar mouse (stimulus 2) was introduced in the testing cage.

As shown in Fig. 5, during the first trials, the WT mice spent about 80% of the time engaging interaction with the mouse stimulus 1, but in the fourth trial there is a reduction of interactions (50% of total time). Finally, in the last trial the time spent for the interaction increased again, showing that WT mice recognized the presence of a new mouse (stimulus 2).

Fmr1 KO mice, in contrast, during the first trial spent half of time ignoring the mouse stimulus 1 and this time increased in the next trial (less of 20% of time spent for interaction). In the same way, during the last trial, in presence of the stimulus 2, *Fmr1* KO mice did not spend more than 40% of time interacting with the new mouse, showing inability to distinguish the unfamiliar mouse.

When *Fmr1* KO mice were therapeutically treated with AF3581 and trofinetide, a redistribution in the time spent interacting with the stimuli was observed. In fact, as reported in Fig. 5A and B, *Fmr1* KO treated mice (both with AF3581 and trofinetide) spent the 80% of time interacting with the stimulus during the first 2 trials and more than 50% in third and fourth trials. However, during the last trial *Fmr1* KO treated mice are not able to distinguish the unfamiliar mouse.

As described in materials and methods section, we evaluated two social investigation parameters corresponding to two types of interaction: social positive interactions (quiet/friendly social behavior) and fighting (non-natural hostile behavior).

To exclude that the increased social interactions observed in *Fmr1* KO mice treated with AF3581 could correspond to an increase of hostile behaviors, we evaluated the time spent in social positive interactions and fighting in all trials for each group (Fig. 6A and B). As reported in the representative Fig. 6C, WT (both vehicle and AF3581 groups) spent more time in social positive interactions and only the 20% of total time was spent in hostile behavior; instead in *Fmr1* KO vehicle group, the fighting-time was more than 40% of the total interactions. Chronic administration of our GSK3 inhibitor increased friendly social behavior and reduced the aggressiveness in *Fmr1* KO mice (less than 20% of total time was spent in hostile behavior), confirming that AF3581 is able to revert social deficits typical of FXS.

Discussion

The present study was designed to explore the therapeutic potential of the GSK3 inhibitor AF3581 for FXS. The compound was originated from our previous medicinal chemistry effort to identify innovative GSK3 inhibitors and represents a significant improvement of *druggability* over the existing ones [21].

To test our compound, we selected an *in vivo* FXS model that is able to mimic the behavioral deficits observed in FXS patients. *Fmr1* deficient mice represent the main preclinical model for this disease because of the manifestation of the major FXS-related behavioral phenotypes [24; 25]. Individuals with FXS, in addition to the (well documented) intellectual disability, have other several abnormalities which may be critical to the overall quality of life, including: hyperactivity/hyperkinesis, mania and obsessive-compulsive behavior, irritability and aggressiveness, hypersensitivity to many different sensory stimuli [32; 33]. Among these many interesting phenotypes, we focused the study of AF3581 on three different phenotypic traits with a battery of behavioral tests: open field (to evaluate the hyperactivity), habituation/dishabituation social interaction test (for unsocial behavior), and acoustic startle response test (for sensorimotor deficits).

The first main finding of the study was that the GSK3 inhibitor AF3581 fully prevents hyperactivity, sensorimotor deficits and the unsocial behavior observed in *Fmr*1 KO mice.

The protective efficacy of our compound was firstly confirmed in the open field test. In particular, in *Fmr*1 KO mice, well-known to have a hyperactive behavior [24], the chronic AF3581 treatment induced a significant reduction of locomotor activity. This therapeutic effect was observed only after chronic treatment, while a

single dose of AF3581 was not able to abolish the hyperactive behavior. Consequently, we conclude that the effect of our compound is not mediated by sedation, but it is likely induced by the long-term molecular modifications caused by the prolonged inhibition of GSK3.

The effect of AF3581 on sensorimotor deficits were evaluated with the acoustic startle response test. FXS patients are hypersensitive to many different sensory stimuli and different animal models for FXS show an extreme response in the presence of an elevated acoustic stimulus, demonstrating abnormal sensorimotor functions [32]. Our results confirmed the significant increased response of *Fmr*1 KO mice compared to WT controls. This anomalous response is completely reverted by the chronic treatment with AF3581.

Difficulty with social interaction is a core diagnostic criterion for ASDs and is associated to FXS. In fact, more than the 60% of FXS subjects exhibits social avoidance [33]. Confirming previous reports [34; 26], our results show the presence of abnormal social behavior in the *Fmr1* KO mice. In particular, we observed a reduced sociability and an inability to distinguish an unfamiliar mouse; this lack of preference for social novelty may be interpreted as an autistic-like deficit in social recognition. This behavior is congruent with the reported social deficits of FXS patients.

The prolonged administration of AF3581 significantly increased the social interactions suggesting a potential effect of this compound on social deficits. To exclude that the effect of our GSK3 inhibitor on social interactions was caused by an increase of hostile behavior, we evaluated the time spent in social positive interactions and in fighting. A tendency to attack a juvenile stimulus mouse (i.e. a social stimulus that normally does not elicit aggressive responses) was found in *Fmr1* KO mice. This behavior resembles the signs of aggressiveness that have been reported in autistic patients, thus *Fmr1* KO mice display inappropriate responses in social situations (as ASDs and FXS subjects). In contrast, *Fmr1* KO AF3581-treated mice spent more time in social positive interaction, reducing the percentage of time dedicated to fight (increase of friendly social behavior and reduction of hostile behavior). These results suggest that the effect of AF3581, was specifically directed towards the friendly social behavior without increasing aggressiveness, confirming the efficacy of this drug in reverting inappropriate social responses.

Finally, it is to be noted that WT mice treated with AF3581 maintained unmodified their behavior in all the performed tests confirming that the efficacy of the drug is not mediated by a sedative effect, but probably by long-term molecular modifications caused by the prolonged inhibition of GSK3.

A second main finding was that the efficacy of AF3581 in different behavioral tests was always comparable or higher than the one of trofinetide, a drug tested for FXS in Phase II clinical trials.

A previous study showed that trofinetide, has a robust effect on hippocampal species-typical behavior (learning and memory, sociability and hyperactivity) in C57BL6 *Fmr1* KO mice possibly by promoting synaptic maturation [35]. Instead, in our study a very low effect of trofinetide was observed on rescuing FXS behavioral phenotypes. This discrepancy with previous studies is probably due to the different tests performed (open field and startle response are hippocampal-independents behavioral tests) or to the mouse strain variability (e.g. different startle response was observed in *Fmr1* KO mice with various background [17]).

Contrary to trofinetide effect, our results suggest a global stabilizing efficacy of AF3581 on all symptoms, both related or unrelated to hippocampal species-typical behavior.

Recently, also lithium, the classical treatment for bipolar disorder, has proven to be beneficial for a surprisingly large number of different phenotypes in FXS mice and in a pilot trial with FXS patients [30; 36]. Previous studies demonstrated that chronic lithium administration, increasing inhibitory phosphorylation of GSK3, ameliorates different behavioral deficits: it reduces audiogenic seizure activity and improved performance on open field test and social interactions in FXS murine models [17; 37; 38]. However, the suspension of lithium treatment has led to the reemergence of the FXS phenotype in *Fmr*1 KO mice [39]. Consequently, lithium would have to be chronically administered to patients for the duration of the lifespan. Although, lithium has proven to be beneficial in FXS mice and in humans [30; 36], it cannot be freely used due to substantial safety and tolerability issues possibly because also interact with many other mechanisms unrelated to GSK3. In particular, lithium can be highly toxic and may not be feasible as a prophylactic or therapeutic agent for pregnant mothers or young children [40; 41]. GSK3 inhibitors may prove to be more viable and safe therapeutic options in the future. In this study, we demonstrated that AF3581, a novel potent GSK3 inhibitor, could exert its efficacy in FXS and could become a new and promising candidate of a new class of drugs.

The mechanism by which GSK3 inhibition could be effective in FXS is still rather unclear. GSK3 regulates the canonical Wnt pathway, preventing βcatenin translocation to the nucleus to induce gene transcription [28].

The p-βcatenin increase observed in our FXS murine model (first in tissues and then in primary neuronal cultures), confirms the already reported elevated GSK3 activity and is consistent with a dysregulation of βcatenin signaling in *Fmr1* KO mice.

AF3581 was able to significantly reduce the p-βcatenin accumulation in *Fmr1* KO neurons, rescuing the basal activity of the kinase, thus bringing back to normal regulation of βcatenin signaling.

Moreover, it has to be reported that the βcatenin reduction seems to be correlated to some autistic-like behaviors observed also in FXS. Indeed, a previous study revealed that conditional knockout mice with mutations in βcatenin (corresponding to *CTNNB1*) of parvalbumin interneurons show cognitive deficit and autistic-like behaviors, suggesting that βcatenin signaling dysregulation is involved in principal autistic-like phenotypes [42].

On these bases, we speculated that the GSK3 overactivation, inducing an abnormal βcatenin degradation, could promote the onset of autistic-like behaviors in FXS. The rescue of behavioral deficits (hyperactivity, sensorimotor deficit and unsocial behavior) on *Fmr1* KO mice by our GSK3 inhibitor AF3581, sustains our hypothesis. Previous studies have indicated that the loss of both GSK3 paralogues, GSK3α and GSK3β, is required to increase βcatenin levels [7, 43]. Despite, AF3581 showed a slight preference for β over α isoform (2-3 fold selectivity) in *in vitro* assays using purified protein, it is quite probable that in a chronic treatment, AF3581 is able to inhibit efficiently both isozymes. This is consistent with the suppression of different behavioral deficits modulated by GSK3α and GSK3β activity [44] in *Fmr1* KO mice treated with AF3581. In particular, social behavior is probably influenced by the activity of both isoforms, but in different manner: GSK3α null mice exhibit more aggressive behavior and reduced social interaction, while the selective loss of GSK3β in the forebrain pyramidal neurons produced anxiolytic and pro-social effects [45]. On these bases, we speculate that the increase of friendly social behavior and the reduction of hostile behavior induced by our compound in *Fmr1* KO mice might be mediated by GSK3β inhibition. In addition, the reduction of sensory-hypersensitivity observed in *Fmr1* KO mice treated with AF3581, in accordance with a recent paper reporting that the selective inhibition of GSK3α corrects excessive protein synthesis and ameliorates the susceptibility to audiogenic seizures in a murine model of FXS [19], confirm the inhibition of GSK3α in a chronic administration of our compound. In summary, findings described in the present study confirm the crucial role of the GSK3 (probably by the modulation of βcatenin signaling) in specific behavioral phenotypes associated

with FXS. Moreover, the widespread therapeutic efficacy demonstrated by the novel potent GSK3 inhibitor AF3581 on all behavioral deficits (thanks to the inhibition of both GSKα and GSK3β isoforms), shows its superiority respect to current medications for FXS, suggesting that it could become a new and promising candidate of a new class of disease-modifying drugs for FXS and related ASDs.

Materials and Methods

Animals and drug treatment

Adult male FVB/NJ wild type mice (WT) and *Fmr1* knockout mice (*Fmr*1 KO) were acquired from Jackson Laboratories (Bar Harbor, ME) and Charles River Laboratories (Italia, Calco, Italy), respectively. Groups of 5 mice were housed by strain in clear polypropylene cages (30 × 19 × 13 cm L × W × H) with food and water *ad libitum* and allowed for two weeks to habituate in a climate-controlled animal facility (21±2°C) and maintained on a 12-hour light/dark cycle, before beginning experimentation. Tests were performed during the light phase (light on: 7 a.m. - 7 p.m.). All experiments were carried out in accordance with the guidelines established by the European Communities Council Directive (Directive 2010/63/EU of 22 September 2010) and approved by the National Council on Animal Care of the Italian Ministry of Health. All efforts were made to minimize animal suffering and to use the minimal number of animals required to produce reliable results. Mice were 3 months of age at the time of data collection. After the behavioral test session, mice were decapitated and tissues were rapidly collected and frozen.

Drugs: Mice were treated i.p. with saline (vehicle) or trofinetide (100 mg/kg dissolved in saline solution; synthesized by C4T) once a day for 30 days or AF3581 (10 mg/kg dissolved in PEG 400/Tween 80/Saline solution at 10/10/80% in volume; synthesized by Angelini Pharma S.p.A.) twice a day for 21 days. As reported by Furlotti et al. (2015), the dose of 10 mg/kg by i.p. route of AF3581 displayed high exposures in plasma and enough brain penetration [21]. Then, Capurro et al. (2020) showed that the dose of 10 mg/kg i.p., twice a day, should provide a sufficient coverage to elicit CNS mediated effects [22].

At the end of the treatment, mice were tested behaviorally to evaluate locomotor activity, social performance and sensorimotor impairments. Animals were sacrificed 24 hours after the last drug administration.

Primary neuronal culture

Primary neurons were isolated from the cortex of both male and female WT and *Fmr1* KO mouse pups at postnatal day 0. For the isolation and culture of cortical neurons, we followed the protocol of Beaudoin et al. (2012) [45]. Briefly, cortices were extracted and collected into dissociation medium (HBSS Ca^{2+} and Mg^{2+} free, sodium pyruvate 100 mM, glucose 45%, 1M Hepes). After three washes with fresh dissection medium, the tissues were suspended in trypsin solution (0.25%) and incubated at 37°C in a water bath for 20 min. After the removal of trypsin, DNAse solution was added for 5 min and rapidly removed with three wash with dissociation medium. Later, cortices were washed three times with prewarmed plating media (MEM Eagle's with Earle's BSS, glucose 45%, sodium pyruvate 100mM, fetal bovine serum 10%, 1M Hepes, L-glutamine 200 mM, Penicillin/Streptomycin) before being triturated 8–10 times. The suspension was strained and cells were plated in 6-well dish (1.5 x 10^6 cells/well). An average of 3 pups was required to have sufficient number of cells for one experiment. Media were changed to maintenance medium (Neurobasal MED SFM, B27 supplement, L-glutamine 200 mM, Penicillin/Streptomycin) 4 hours post-seeding. Primary neurons were maintained at 37°C, 5% CO2. Two days after plating, cytosine arabinoside (araC; 1-b-d-arabinofuranosylcytosine) was added at a final concentration of 2 μM to inhibit the proliferation of dividing non-neuronal cells.

Analysis of phosphorylated βcatenin expression

Neuronal cell culture treatment with MG132

For determination of p-βcatenin accumulation in neuronal cell cultures**,** after 7 days of culture, medium was exchanged with 3 ml of fresh medium containing DMSO (0.25%) or AF3581 2.5 μM dissolved in DMSO (0.25% final concentration). After 30 min, MG132 (M7449, Sigma Aldrich) was added at 10 μM (0.25% DMSO) in both wells treated with DMSO and with AF3581. Wells with DMSO only (0.5%) were used as controls. After 6 hours from MG132 addition, cells were harvested and cell pellets were stored at -80 °C until used.

Protein extracts preparation

For mice tissue collection adult WT and *Fmr1* KO mice were sacrificed with CO_2 exposure, tissues were immediately collected, snap frozen in liquid nitrogen and stored at -80 °C until used. For neuronal cells analysis, after treatment, cells were harvested with trypsin, washed twice with Phosphate Buffered Saline pH 7.4 and pellets were stored at -80 °C until used.

Tissues and neuronal cell cultures were homogenized in lysis buffer [50 mM Tris Hcl pH 8, 150 mM NaCl, 2mM EDTA, 1% TRITON-X100] added with protease and phosphatase inhibitors cocktail. Lysates were centrifuged 10 min at 10.000xg to remove tissue debris. Protein content in the supernatant was quantified by bicinchoninic acid (BCA) assay.

Western blot

Lysates (30μg) were subjected to SDS-PAGE and resolved samples were transferred into nitrocellulose membranes that were blocked with 5% non-fat dry milk powder in PBS/0.1% and Tween 20. The following antibodies were used: FMRP #4317, β-Catenin #2698, Phospho-βCatenin (Ser33/37/Thr41) #9561 (all from Cell Signaling), GAPDH #MA5-15738 (ThermoFisher Scientific), and βactin #A2228 (Sigma Aldrich).

Behavioral procedures

Behavioral tests commenced at the last week of treatments, as follows: starting on day 1, the open field test was performed, followed on day 3 by habituation/dishabituation social interaction test with two juvenile male mice stimuli, and on day 7 by startle response test.

Locomotor activity (Open field test)

The open field test is suitable for assessing locomotor activity of mice. To measure the spontaneous motor activity, mice were placed individually in the center of an open field apparatus and the locomotor activity was measured in terms of the total distance covered. It uses an open field plexiglas chamber that measures 40 cm long x 40 cm wide × 40 cm high (Ugo Basile Srl, Gemonio, Italy). The animal is placed into the center of the apparatus and its movements are recorded for 60 min. After the experiment is completed, the mouse was removed from the arena, placed back in the home cage and a video tracking system (ANY-maze, Stoelting Co.) analyzes the movements of the animal over time. The program can detect the animal's behavior in zones, the periods when the animal is immobile, the sequences of movements between the different parts of the open

field – (for example, transitioning across the center, or walking right round the border), what direction the animal is facing - (for example, facing towards the center or the towards the walls).

Sensorimotor functions (Acoustic startle response test)

Mice were tested in Startle Response/PPI test system chambers (TSE Systems GmbH, Germany) using standard methods as described by Papaleo et al. (2008) [31]. Test session began by placing the mouse in the plexiglas holding cylinder for a 5 min acclimation period. Over the next 10.5 min, mice were presented with each of seven trial types across six discrete blocks of trials for a total of 42 trials. The order in which trial types were presented was randomized within each block. The interval between trials was 10–20 seconds. One trial type measured the response to no stimulus (baseline movement), and another presented the startle stimulus alone (acoustic amplitude), which was a 120 dB sound burst (for 40 milliseconds). The maximum startle amplitude was the dependent variable. A background level of 70 dB white noise was maintained over the duration of the test session and the startle response was automatically (TSE Startle Response System) measured by normalizing the mouse movements of the "startle" session over the "no stimulus" one.

Social behavior (Habituation/dishabituation social interaction test)

Mice were tested in 2150E Tecniplast cages (35.5 × 23.5 × 19 cm) lightly illuminated (5±1 lux) and video-recorded using a Unibrain Fire-i digital camera. The video camera was mounted facing the front of the cage to record the session for subsequent scoring of social investigation parameters. Male mice were individually placed in the testing cage 1 hour prior to the testing (adaptation period). No previous singlehousing manipulation was adopted to avoid any instauration of home-cage territory and aggressive behaviors. Testing began when a stimulus male mouse was introduced into the testing cage for a 1 min interaction. At the end of the 1 min trial, we removed the stimulus animal and returned it to an individual holding cage. This sequence was repeated for 4 trials with 3 min inter-trial intervals introducing the same stimulus mouse in all 4 trials. In a fifth 'dishabituation' trial, a new unfamiliar stimulus mouse was introduced in the testing cage. Videos of behaviors were recorded and subsequently scored offline to evaluate the total time of social interactions. Usually, the interactions between two mice are quiet/friendly social interaction and non-natural hostile behaviors (such as abnormal aggressiveness and attacks towards the intruder accompanied by strong fighting

were seen against the intruder that normally would have not occurred). On these bases, we identified and evaluated two social investigation parameters: social positive behavior and fighting.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using Prism-8 software (version 8.00, Graph Pad, San Diego, CA). Two-way analysis of variance (ANOVA, multiple comparisons) or paired T-test analysis were used as a statistical analysis, as appropriate: Two-way ANOVA followed by Bonferroni post hoc test was used for all behavioral tests. The unpaired T-test analysis was used for the biomolecular analysis. Results were considered significant at $p<0.05$.

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Conflict of Interest Statement

There is no conflict of interest.

Author contributions statement

B.G., L.D., C.M. and F.P.D. conceived the experiment(s), P.F.P., M.C. and ER conducted the experiment(s) and analyzed the results. P.F.P and A.R. wrote and reviewed the manuscript.

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Legends to Figures

Fig. 1. *Fmr1* KO mice show an increased GSK3 activity in peripheral tissue. GSK3 activity in WT and *Fmr1* KO mice liver tissue was evaluated by determining phosphorylated βcatenin (p-βcatenin) expression levels by Western blot (A) and densitometry analysis of the obtained protein bands (B). An anti-FMRP antibody was used to check for gene knock-out and GAPDH evaluation was used for samples' normalization. Phosphorylation rate of βcatenin was expressed as ratio of p-βcatenin to total βcatenin. Mean ± SEM values are reported (n=4-6), *P<0.5, **P<0.01 (unpaired T-test analysis).

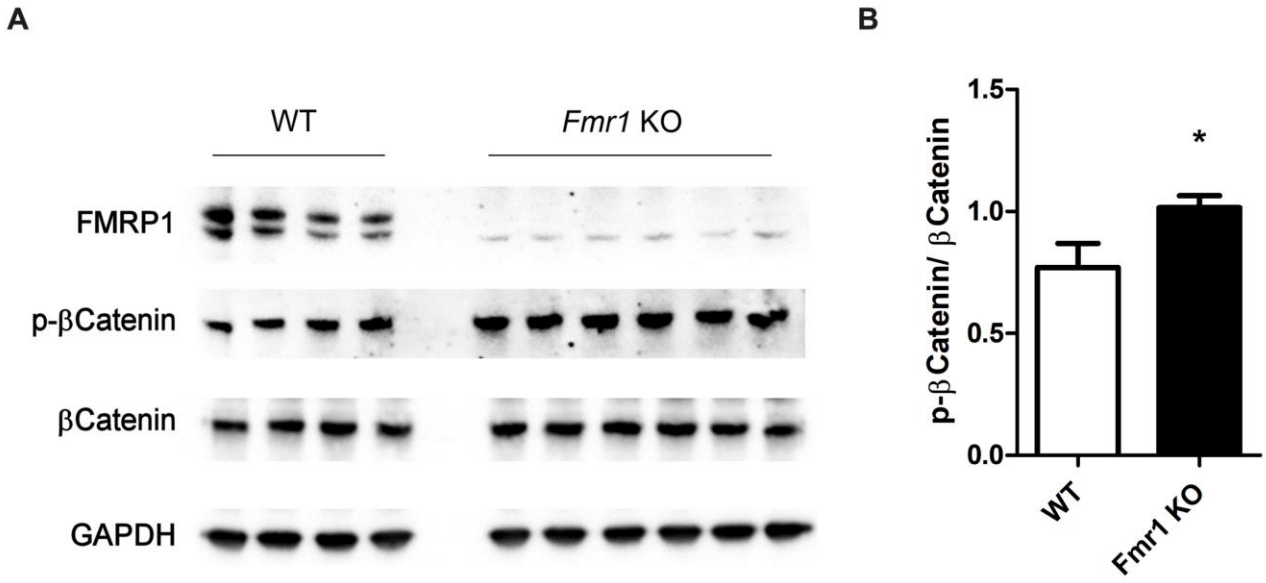


Fig. 2. Primary neuronal cell cultures from *Fmr1* KO mice show an increased GSK3 activity. Western blot analysis of phosphorylated βcatenin (p-βcatenin) in neuronal cells derived from WT and *Fmr1* KO mice brain cortex cultivated for 6 hours in presence of the proteasome inhibitor MG132 (panel A). DMSO was used as control of βcatenin basal phosphorylation. AF3581 (2.5 mM) was added to block GSK3 showing its exclusive contribution to βcatenin phosphorylation. An anti-FMRP antibody was used to check for gene knock-out and βactin was used for samples' normalization. Gel shows 2 out of 4 independent experiments. Densitometry analysis of p-βcatenin western blot bands (panel B). Data are expressed as percentage of WT + DMSO. Mean ± SEM values are reported, n=4. Statistical analysis: Two-way ANOVA, (significant effect of treatment P<0,0001), Bonferroni post-hoc test *P<0.5$_{\text{KO + DMSO vs KO + MG132}}$; **P<0.01$_{\text{KO + MG132 + AF3581 vs KO + MG132}}$.

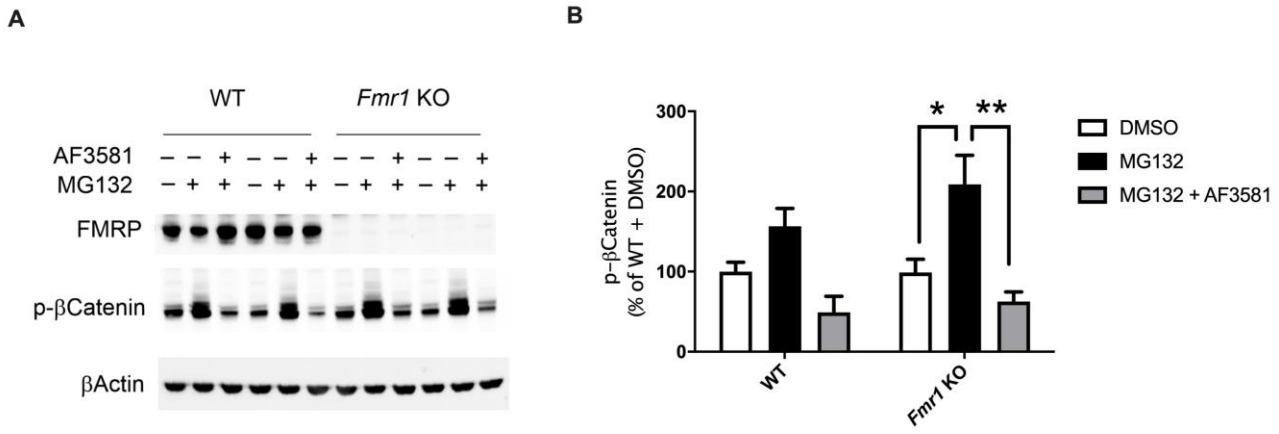


Fig. 3. AF3581 chronic treatment reduces hyperactive behavior in *Fmr1* KO mice (Open field test). Effect of AF3581 (panel A [Two-way ANOVA, significant effect of genotype (P<0.0001), treatment (P=0.05) and interaction (P=0.02), post-hoc test $P_{WT\ VEH\ +\ KO\ VEH}$<0.0001; $P_{KO\ VEH\ +\ KO\ AF3581}$=0.0079]) and trofinetide (TROF) (panel B [Two-way ANOVA, significant effect of genotype (P=0.0044), treatment (P=0.9) and interaction (P=0.033), post-hoc test $P_{WT\ VEH\ +\ KO\ VEH}$<0.0001]) on the locomotor activity of WT and *Fmr1* KO mice after chronic treatments (AF3581 was administered twice a day at 10 mg/kg for 21 days; trofinetide was administered once a day at 100 mg/kg for 30 days); effect of AF3581 on the locomotor activity of WT and *Fmr1* KO mice after acute treatment (panel C) with AF3581 (a single dose of 10 mg/kg administrated 1 hour before the test) [Two-way ANOVA, significant effect of genotype (P=0.0009), treatment (P=0.76) and interaction (P=0.71), post-hoc test $P_{WT\ VEH\ +\ KO\ VEH}$=0.014].

As measure of locomotor activity, was evaluated the total distance travelled (m). Data are the mean ± SEM (n=8) of the distance travelled after 60 min recording. Symbols represent significant changes from *Fmr1* KO vehicle group (**P<0.01, ***P<0.001). Statistical analysis: Two-way ANOVA followed by the Bonferroni post-hoc test.

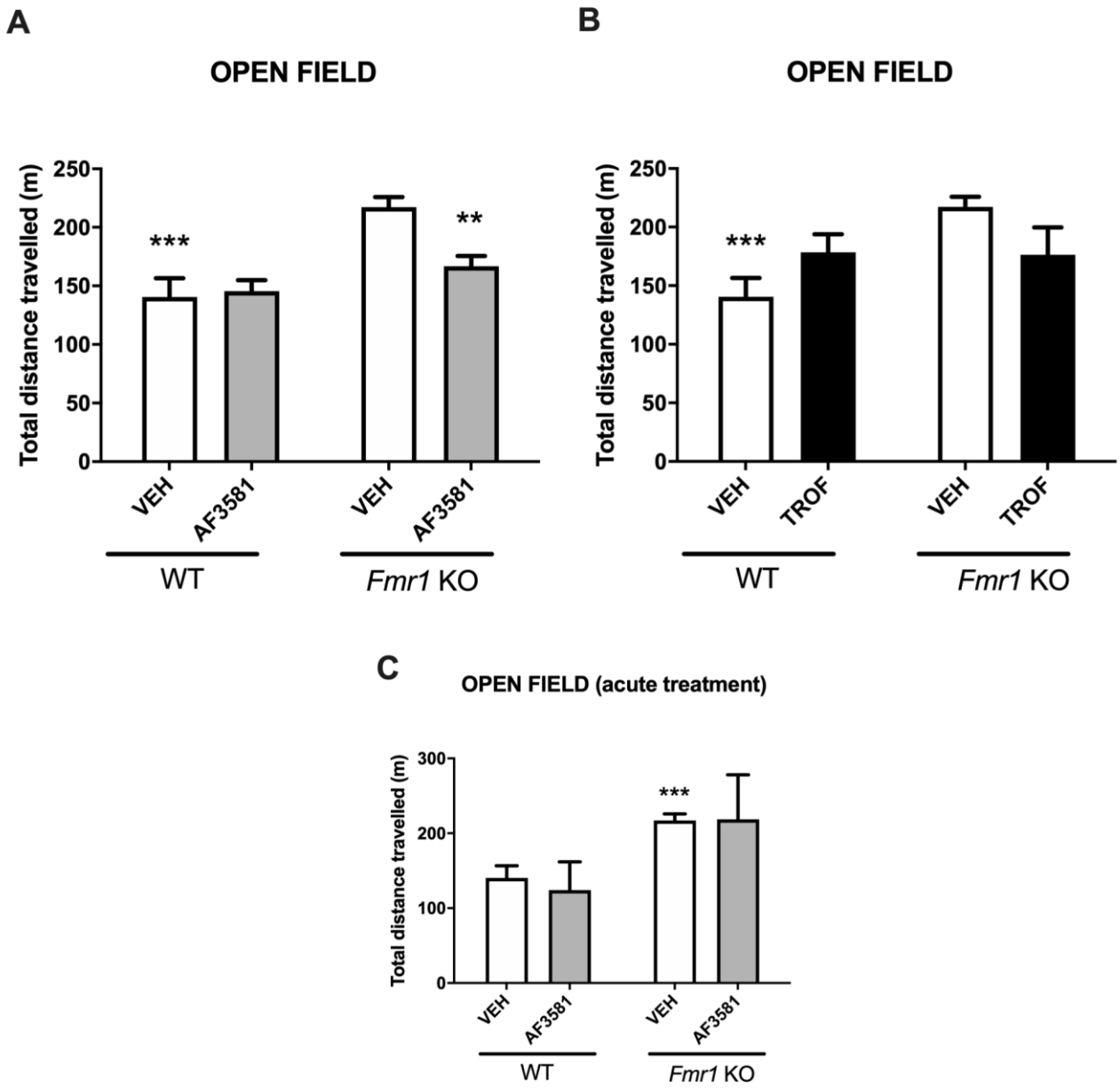


Fig. 4. AF3581 chronic treatment reverts sensorimotor deficit in *Fmr1* KO mice (Acoustic startle response test). Effect of chronic administration of AF3581 (panel A [Two-way ANOVA, significant effect of stimuli (P<0.0001), groups (P=0.0002) and interaction (P<0.0001), post-hoc test $P_{WT\ VEH\ +\ KO\ VEH}$=0.041; $P_{KO\ VEH+\ KO\ AF3581}$<0.0001]) or trofinetide (panel B [Two-way ANOVA, significant effect of stimuli (P<0.0001), groups (P=0.04) and interaction (P=0.022), post-hoc test $P_{WT\ VEH\ +\ KO\ VEH}$=0.068]) on acoustic startle response in WT and *Fmr1* KO mice. The sensorimotor deficit was evaluated measuring the mouse response first in absence ("no stimulus") and then in presence ("startle") of an elevated acoustic stimulus (120 dB of amplitude and 40 milliseconds of duration). Data are the mean ± SEM (n=8) of startle amplitude displayed by WT and *Fmr1* KO mice after the presentation of no stimulus or a 120 dB stimulus (Startle). Symbols represent significant changes from *Fmr1* KO vehicle group (**P<0.01, ***P<0.001). Statistical analysis: Two-way ANOVA followed by the Bonferroni post-hoc test.

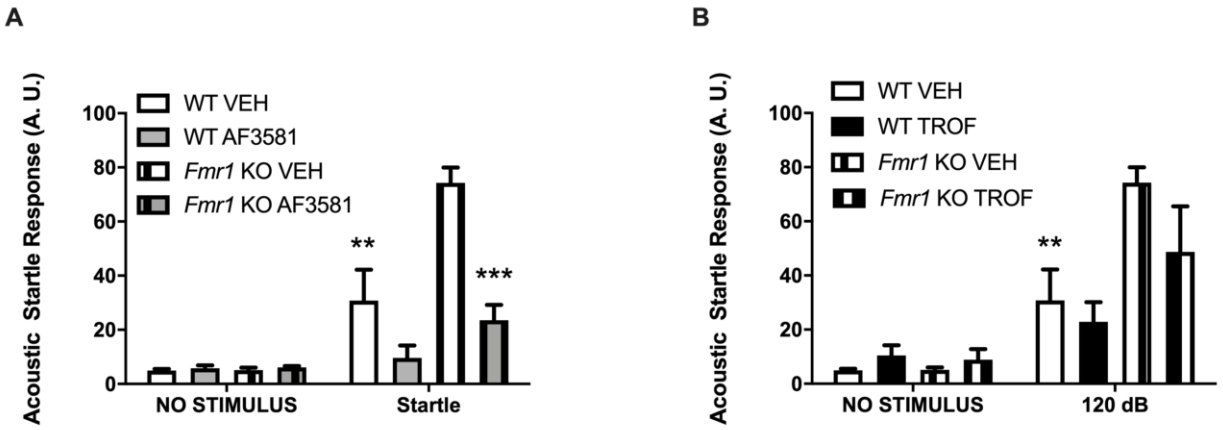


Fig. 5. AF3581 chronic treatment reverts social deficits in *Fmr1* KO mice. (Habituation/dishabituation social interaction test). Effects of repeated assumption of AF3581 (panel A [Two-way ANOVA, significant effect of trials (P=0.0014), groups (P=0.013) and interaction (P=0.031)]) or trofinetide (panel B [Two-way ANOVA, significant effect of trials (P=0.0079), groups (P=0.021) and interaction (P=0.067)]) on habituation/dishabituation social interaction test. This test evaluates social interactions and recognition of an unfamiliar mouse. After the adaptation period, the test mouse was exposed to an unknown mouse (stimulus 1) for a test time of 1 min. This sequence was repeated for 4 trials with 3 min inter-trial intervals introducing the same stimulus mouse in all 4 trials. In a fifth dishabituation trial, a new unfamiliar mouse (stimulus 2) was introduced in the testing cage. Videos of behaviors were recorded and subsequently scored offline to evaluate the total time of social interactions (data are the mean ± SEM, n=8). Statistical analysis: Two-way Repeated Measures ANOVA followed by the Bonferroni post-hoc test.

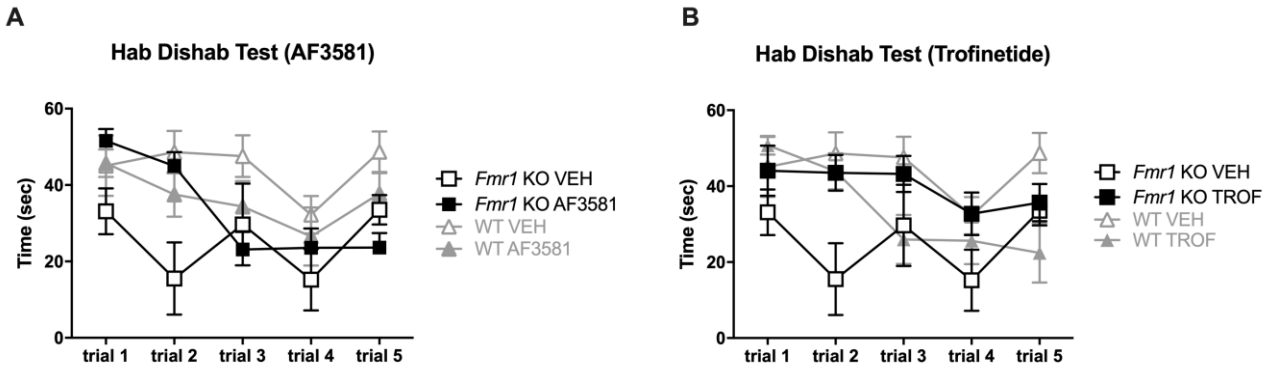
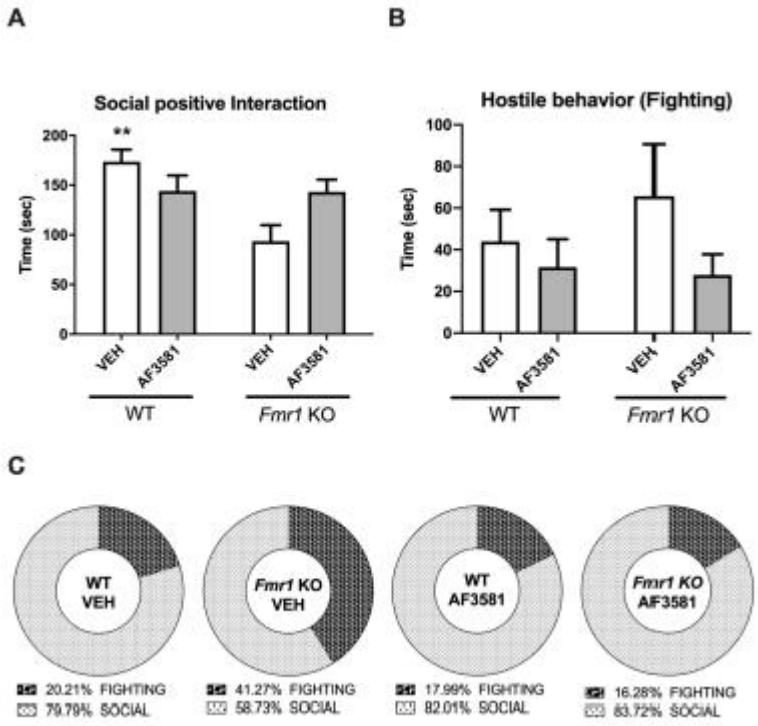
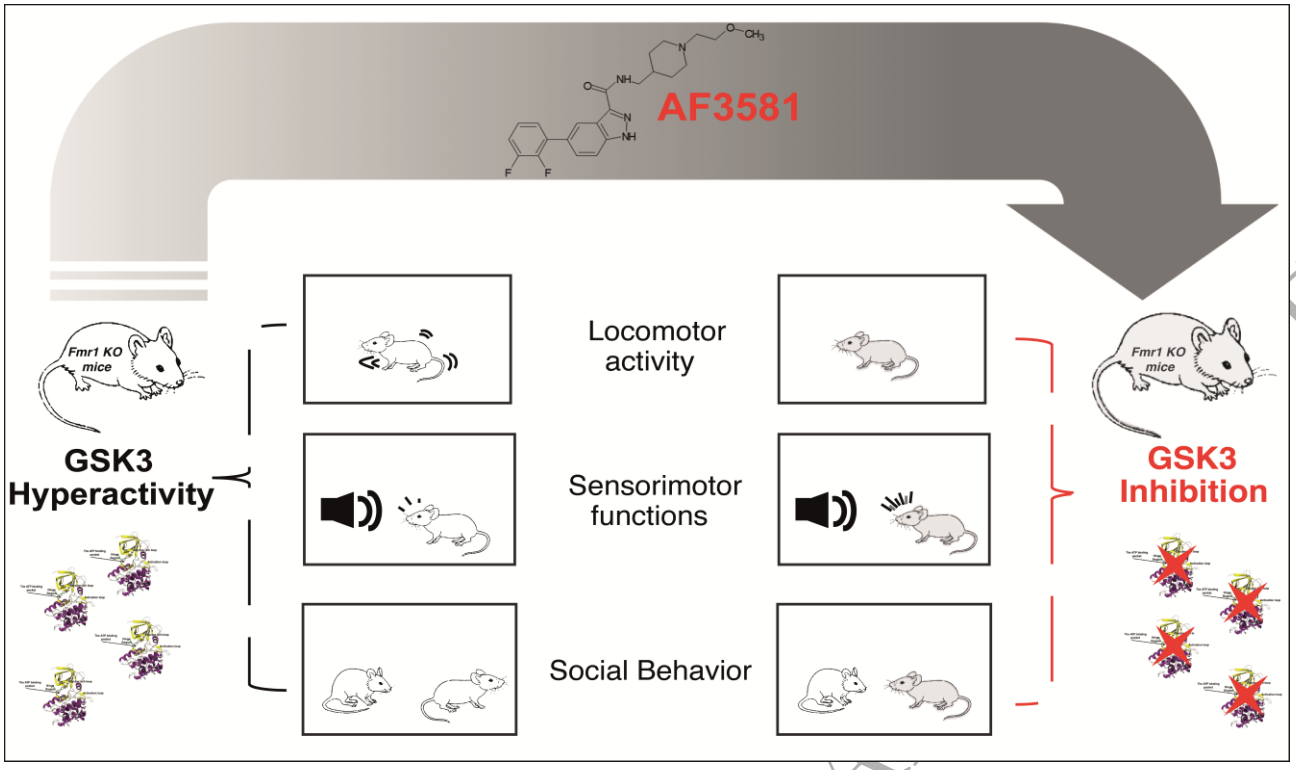


Fig. 6. AF3581 improves positive social interactions in *Fmr1* KO mice. Effects of AF3581 on mice aggressiveness and social positive interaction during social interaction. After the evaluation of total time of social interactions in habituation/dishabituation social interaction test, we classified the interactions between two mice in: quiet / friendly social interaction (social, panel A [Two-way ANOVA, significant effect of genotype (P=0.012), treatment (P=0.5) and interaction (P=0.014), post-hoc test $P_{WT\ VEH\ +\ KO\ VEH}$=0.013]) and non-natural hostile behaviors such as abnormal aggressiveness (fighting, panel B [Two-way ANOVA, no significant effect of genotype (P=0.55), treatment (P=0.11) and interaction (P=0.4)]). In panel C was evaluated the percentage of time spent in positive interaction (social) and of time spent in hostile behavior during each attack (fighting) in all trials for each group (data are the mean ± SEM, n=8). Symbols represent significant changes from *Fmr1* KO vehicle group (**P<0.01). Two-way ANOVA followed by the Bonferroni post-hoc test.





ABBREVIATIONS

ANOVA, analysis of variance;

ASDs, Autism Spectrum Disorders;

CNS, central nervous system;

FMR1, fragile X mental retardation 1 gene;

FMRP, Fragile X Mental Retardation Protein;

FXS, Fragile X Syndrome;

GSK-3 β, glycogen synthase kinase 3 β;

i.p., intraperitoneally;

KO knockout

p-βcatenin, phosphorylated βcatenin;

PK, pharmacokinetic.

WT wild type