HAL
archives-ouvertes.fr

Rescue of fragile X syndrome phenotypes in Fmr1 KO mice by a BKCa channel opener molecule.

Betty Hébert, Susanna Pietropaolo, Sandra Même, Béatrice Laudier, Anthony Laugeray, Nicolas Doisne, Angélique Quartier, Sandrine Lefeuvre, Laurence Got, Dominique Cahard, et al.

▶ **To cite this version:**

Betty Hébert, Susanna Pietropaolo, Sandra Même, Béatrice Laudier, Anthony Laugeray, et al.. Rescue of fragile X syndrome phenotypes in Fmr1 KO mice by a BKCa channel opener molecule.. Orphanet Journal of Rare Diseases, BioMed Central, 2013, pp.124. <inserm-01089760>

HAL Id: inserm-01089760

http://www.hal.inserm.fr/inserm-01089760

Submitted on 2 Dec 2014

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

ORPHANET JOURNAL OF RARE DISEASES

RESEARCH Open Access

Rescue of fragile X syndrome phenotypes in *Fmr1* KO mice by a BKCa channel opener molecule

Betty Hébert[1,2†], Susanna Pietropaolo[3,4†], Sandra Même[5], Béatrice Laudier[1,2,6], Anthony Laugeray[1,2], Nicolas Doisne[1,2], Angélique Quartier[1,2], Sandrine Lefeuvre[6], Laurence Got[6], Dominique Cahard[7], Frédéric Laumonnier[8,9], Wim E Crusio[3,4], Jacques Pichon[1,2], Arnaud Menuet[1,2], Olivier Perche[1,2,6] and Sylvain Briault[1,2,6*]

Abstract

Background: Fragile X Syndrome (FXS) is the most common form of inherited intellectual disability and is also associated with autism spectrum disorders. Previous studies implicated BKCa channels in the neuropathogenesis of FXS, but the main question was whether pharmacological BKCa stimulation would be able to rescue FXS neurobehavioral phenotypes.

Methods and results: We used a selective BKCa channel opener molecule (BMS-204352) to address this issue in *Fmr1* KO mice, modeling the FXS pathophysiology. *In vitro*, acute BMS-204352 treatment (10 μM) restored the abnormal dendritic spine phenotype. *In vivo,* a single injection of BMS-204352 (2 mg/kg) rescued the hippocampal glutamate homeostasis and the behavioral phenotype. Indeed, disturbances in social recognition and interaction, non-social anxiety, and spatial memory were corrected by BMS-204352 in *Fmr1* KO mice.

Conclusion: These results demonstrate that the BKCa channel is a new therapeutic target for FXS. We show that BMS-204352 rescues a broad spectrum of behavioral impairments (social, emotional and cognitive) in an animal model of FXS. This pharmacological molecule might open new ways for FXS therapy.

Keywords: Fragile X Syndrome, BMS-204352, BKCa channel, Sociability, Cognition, Anxiety

Background

Fragile X Syndrome (FXS) is the most common cause of inherited mental deficiency and is associated with autistic features [1]. FXS is caused by a CGG triplet expansion in the *FMR1* gene resulting in the absence of its coding protein, Fragile X Mental Retardation Protein (FMRP). This mRNA-binding protein regulates both localization and translation of specific mRNAs in synaptic regions [2], but also controls synaptic membrane proteins activity through a translation-independent pathway [3]. As a consequence of this synaptic disturbance, a preponderance of long, thin and 'tortuous" dendritic spines in cortex is observed in FXS patients brain [4]. *Fmr1* knock-out (KO) mouse [5], a murine model of human FXS, presents both dendritic spines maturation abnormalities [6] and many behavioral characteristics similar to human FXS, including altered social interaction, occurrence of repetitive behaviors, hyperactivity and cognitive dysfunction [7,8].

In the last decade, a better understanding of the FXS pathophysiology allowed to develop chemical therapeutics targeting specific synaptic components. Numerous studies have revealed that in the absence of FMRP, signaling through group 1 metabotropic glutamate receptors (mGluR 1/5) is disturbed. This dysfunction might underlie either the observed exaggerated synaptic long-term-depression or the insensibility of mGluR 1/5-mediated neuronal/synaptic protein synthesis stimulation [9]. Based on "other active mGluR 1/5 functions", various selective molecules were tested *in vitro* on primary neuron cell cultures and *in vivo* on several behavioral defects in both *Fmr1* KO mice and FXS patients [10-12]. Although promising results were obtained, none of the tested therapeutic agents demonstrated a full effect on FXS behavioral, cognitive and molecular abnormalities. These reports prompt the interest of developing novel therapeutic targets.

* Correspondence: sbriault@cnrs-orleans.fr
†Equal contributors
[1]UMR7355, CNRS, Orléans, France
[2]Experimental and Molecular Immunology and Neurogenetics, University of Orléans, 3b rue de la Férollerie, 45071 Orléans, Cedex 2, France
Full list of author information is available at the end of the article

BioMed Central

© 2014 Hébert et al.; licensee Biomedcentral Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

Recent studies have demonstrated the implication of potassium channels in FXS pathology [13]. Among them, large-conductance Ca^{2+}-activated K^+ channels (BKCa channels, also known as BK or Maxi-K channels), activated by membrane depolarization and increased intracellular Ca^{2+} concentration, are of particular interest because of their control of Ca^{2+} concentration in neurons and regulation of neurotransmitter release such as glutamate [14,15]. Functional BKCa channels are assembled as hetero-octamers of four α- subunits (KCNMA1 protein) and four auxiliary β-subunits, where the β-subunit is a tissue specific regulatory unit [16].

Several data provide convincing evidence that this channel is closely linked to behavioral and cognitive disorders. Our physical mapping of balanced chromosomal aberrations revealed a *KCNMA1* gene disruption in a subject with autism and intellectual deficiency. This gene haploinsufficiency induced a functional defect of BKCa channels that might contribute to neurological symptoms [17]. In addition, a mutation in the *CRBN* gene, an upstream regulator of BKCa channel, has been also associated with autosomal recessive non-syndromic mental retardation [18]. Behavior analysis obtained in mouse in which *Kcnma1* gene was deleted, showed a critical role for the BK channels in mechanisms underlying associative learning [19]. The hypothesis concerning BKCa involvement in FXS neuropathology was first proposed by Liao et al. [20]. In primary neuron cultures from *Fmr1* KO embryos, the authors observed a decreased expression of KCNMA1 protein. This defect suggested that some component of autistic and cognitive disorders seen in FXS might be, in part, due to BKCa channel activity abnormalities. Recently, Deng *et al.* [3] have demonstrated that BKCa current reduction in hippocampal slices results from an FMRP translation-independent effect. In hippocampal pyramidal neurons, and probably in cortical neurons, FMRP regulates action potential duration, neurotransmitter release and short-term plasticity through presynaptic BKCa channels. Based on all these data, we propose BKCa channel as a new pharmacological target in FXS therapy.

For this purpose, we used the fluoro-oxindole BMS-204352 ((3S)-(+)-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2H-indol-2-one), as a selective BKCa channel opener [16,17]. To investigate the therapeutic potential of this molecule, its effect was tested at first *in vitro* using a standardized primary neuron culture protocol focused on the *Fmr1* KO dendritic spines defects [6]. Based on promising *in vitro* results, we investigated its effect in *in vivo* conditions on *Fmr1* KO mice behavioral impairments as well as on metabolites homeostasis at hippocampus level [21]. In accordance with the main human clinical manifestations described in DSM-5 (Diagnostic and Statistical Manual of Mental Disorders), our behavior investigations concerned social/cognitive skills.

Methods

For a full methodological description, see the supplementary content that accompanies the online edition of this article.

Animals

All experiments were done with adult (3–5 months old) male *Fmr1* KO mice and their wild-type (WT) littermates of C57BL/6 J background [5]. *Fmr1* mice were obtained from the colony of the Aquitaine Institute for Cognitive and Integrative Neuroscience in Bordeaux (France). Breeding details were described in Additional file 1: supplementary methods. The present experimental protocol received full review and approval by the regional animal care and use committee (CREEA) prior to conducting the experiments. All possible efforts were made to reduce the number of animals studied and to avoid their suffering.

Neuronal cell cultures and BMS-204352 treatment

Primary neuron cultures were prepared from male WT and *Fmr1* KO mice at embryonic day 15 (E15) as previously described [22]. Neurons were DiI-labeled and dendritic spine maturation were analysed using a protocol adapted from [6]. Experiments and analysis were done as blind to the genotype.

Behavioral assays

The experiments were conducted in two laboratories: The Experimental and Molecular Immunology and Neurogenetics laboratory in Orléans, France (Laboratory A), and The Aquitaine Institute for Cognitive and Integrative Neuroscience in Bordeaux, France (Laboratory B). Mice were bred and maintained in the two laboratories following identical procedures.

In order to assess the social, cognitive and emotional components, we performed a direct social interaction test, the three-chamber test, the Y maze, and the elevated plus maze. The first two behavioral tests, i.e., the direct social interaction test and the three-chamber test for sociability and social recognition were conducted in parallel in both laboratories. This experimental strategy was adopted in order to assess the robustness and replicability of the results obtained. The last two tests, i.e., the Y maze for spontaneous alternation and the elevated plus maze were carried out in Laboratory B and Laboratory A, respectively. In order to minimize the number of animals studied, the same mice were used during the first three behavioral tests. Experiments and analysis were done as blind to the genotype.

All behavioral tests are described in Additional file 1: (Methods and Supplementary Results).

Drug administration

The effective dose of BMS-204352 was chosen based on previously published data [23,24]. BMS-204352 (2 mg/kg) diluted in the vehicle solution (DMSO 1/80; Tween 80 1/80; 0.9% NaCl) was administered by a 10 ml/kg single intraperitoneal (i.p) injection. Behavioral tests were performed at the maximal BMS-204352 brain concentration, i.e., 30 min after injection (Details appear in Additional file 1: Methods and Supplementary Results obtained by LC-MS/MS method, Additional file 1: Figure S1).

Magnetic Resonance Spectroscopy (MRS)

MRS was realized as described previously with slight modifications [25] in order to quantify brain metabolites: glutamate, myo-inositol, N-Acetyl-Aspartate, taurine and lactate. Ten *Fmr1* KO and ten WT mice, which were not used in the behavioral tests, were included in the study. Metabolites concentrations are represented in arbitrary unit (AU), after normalization by Creatine/Phosphocreatine. Method is described in Additional file 1.

Statistical analysis

For behavioral tests, data were analysed using three-way ANOVA with genotype, treatment, and laboratory as main factors for the three compartment and social interaction tests, and two-way ANOVA with genotype and treatment for the Y maze and elevated plus maze. Within-subject factors (e.g., contact area) were included when appropriate. Fisher PLSD's *post-hoc* comparisons were used when a statistically significant main effect or interaction was detected ($p < 0.05$). For *in vitro* analyses, data were analysed using two-way ANOVA with genotype and treatment as main effects followed by Fisher PLSD's *post-hoc* comparisons. All statistical analyses were done using Statistica 8 (StatSoft). For MRS, metabolites concentrations obtained in WT or *Fmr1* KO mice were compared using t-test or Mann–Whitney tests when non-parametric analysis was required. Treatment efficiency was evaluated by Wilcoxon tests.

Results

BMS-204352 reverses dendritic spine abnormal phenotype

Increased dendritic spine density and length was reported in *post-mortem* analysis of FXS patients brain tissues [4] and observed in *Fmr1* KO mice *in vivo* [26] as well as *in vitro* on primary neuron cultures [6]. Vehicle-treated *Fmr1* KO neurons showed a significantly higher filopodia length (3.00 ± 0.15 μm *vs* 1.67 ± 0.2 μm, $p < 0.0001$) and density (0.93 ± 0.11 nbr/10 μm *vs* 0.15 ± 0.04 nbr/10 μm, $p < 0.001$) compared to vehicle-treated WT neurons (Figure 1). Acute treatment (4 hrs) with BMS-204352 10 μM corrected the *Fmr1* KO dendritic spine phenotype, whereas, BMS-204352 5 μM had no significant effect on *Fmr1* KO neurons. Indeed, BMS-204352 10 μM significantly reduced filopodia length (1.72 ± 0.12 μm, $p < 0.01$) and density (0.21 ± 0.04 nbr/10 μm, $p < 0.01$) of *Fmr1* KO neurons. In WT neurons, BMS-204352 (5 μM or 10 μM) acute treatment had no significant effect on the dendritic spine length or density.

BMS-204352 reverses impaired social behavioral phenotype

Impairments in social interactions are core symptoms in FXS patients [1]. The assessment of *Fmr1* KO direct social interaction and BMS-204352 effect was conducted in two different laboratories (Laboratories A and B). A non-significant effect of laboratory [$F(1,77) = 1.12$, NS] and of its interactions with genotype [$F(1,77) = 0.62$, NS], treatment [$F(1,77) = 0.39$, NS] and both factors [$F(1,77) < 1$, NS] was observed. On the other side, a significant interaction of genotype and treatment was noticed [$F(1,77) = 10.66$, $p < 0.01$]: regardless the laboratory, vehicle-treated *Fmr1* KO showed significantly reduced social interactions with a stimulus female mouse compared to vehicle-treated WT animals [*post-hoc*, $p < 0.01$], and this reduced social investigation was corrected by BMS-204352 [*post-hoc*, $p < 0.01$], while a tendency to a negative effect of the treatment was observed in WT [*post-hoc*, $p = 0.06$] (Figure 2a). Indeed, a single BMS-204352 2 mg/kg injection in *Fmr1* KO mice was able to correct their deficits in social interactions, making them indistinguishable from WT mice. These results were confirmed in both laboratories, despite the obvious differences in environmental factors, i.e., experimenters (Additional file 1: Figure S2a-b).

To go further, we performed a second test in order to assay sociability, the three-chamber test. In the habituation phase (trial 1), all mice did not show a preference for any compartment or contact area in both laboratories (Additional file 1: Figure S2c-d). No difference between genotypes and treatments was observed for locomotor activity (Additional file 1: Figure S2g).

In trial 2, all mice preferentially explored the contact area containing the stimulus mouse compared to the one with the object, [main effect of contact area: $F(1,112) = 54.11$, $p < 0.0001$; Figure 2c], without difference between genotypes and treatments. A main effect of laboratories was observed [$F(1,112) = 6.71$, $p < 0.05$] since mice from Laboratory B spent more time in both contact areas, without interaction between genotype and treatments (Additional file 1: Figure S2e). These results confirmed the lack of deficits in sociability previously described in *Fmr1* KO mice in this test [8,27-29]. Locomotor activity was similar in *Fmr1* KO and WT mice [main effect of genotype: $F(1,112) = 2.25$, NS ; Additional file 1: Figure S2h].

In trial 3, the social recognition trial, WT mice preferred to spent time in contact with the novel compared to the familiar mouse [main effect of contact area:

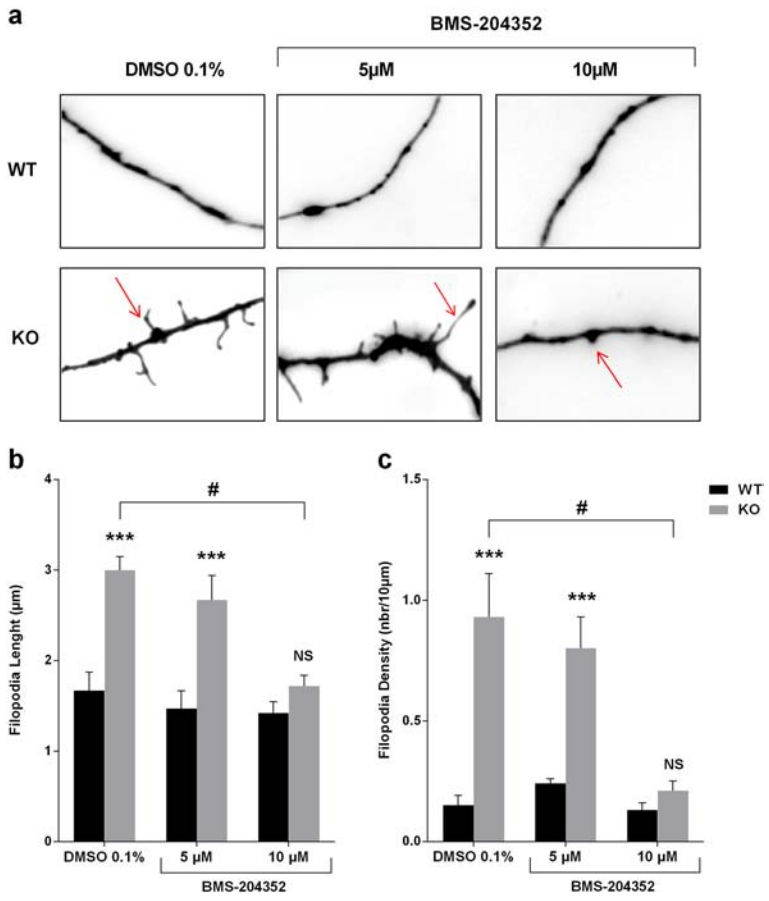

Figure 1 BMS-204352 at 10 μM rescues dendrite spines maturation in Fmr1 KO neurons cultures. (a) Representative pictures of neuron dendrites in different culture conditions with BMS-204352 (5 or 10 μM) or with only its vehicle (DMSO 0.1%). Red arrows indicate filopodia. Scale bar = 10 μm **(b)** Filopodia length (μm) and **(c)** density (nbr/10 μm) were investigated in each condition. A two-way ANOVA revealed that vehicle-treated *Fmr1* KO neurons showed a significantly higher filopodia length and density compared to vehicle-treated WT neurons. Acute treatment (4 hrs) with BMS-204352 10 μM corrected the *Fmr1* KO dendritic spine phenotype, whereas, BMS-204352 5 μM had no significant effect on *Fmr1* KO neurons. NS, not significant; ***p < 0.001 for genotype comparison; #p < 0.05 for treatment comparison; *n* = 60 neurons (from 10 mice) in all groups. Data represent mean ± s.e.m.

$F(1,112) = 8.81$, $p < 0.01$], but this effect was modulated by both genotype and treatment [interaction genotype × treatment × contact area: $F(1,112) = 10.02$, $p < 0.01$]. Indeed, separate analyses in each group revealed that vehicle-treated WT mice showed a marked preference for social novelty, while this was absent in vehicle-treated *Fmr1* KO mice [interaction genotype x contact area: $F(1,74) = 7.31$, $p < 0.01$]. This deficit in social recognition was abolished by BMS-204352 treatment in *Fmr1* KO mice [interaction contact area x treatment: $F(1,65) = 4.74$, $p < 0.05$], as shown by the heat map illustration (Figure 2b), although a tendency to a negative effect was observed in WT mice [interaction contact area × treatment: $F(1,51) = 3.27$, $p = 0.08$; Figure 2d]. This negative effect in the WT mice was the consequence of an increase time spent in contact to familiar mouse *versus* a decrease time spent in contact to novel mouse (Figure 2d). All these effects were equivalently observed in both laboratories [all interactions with laboratory, NS; data not shown]. As in trial 2, a main effect of laboratory was observed [$F(1,112) = 6.11$, $p < 0.05$], without any consequences on the area preference or the differences between genotypes and treatments (Additional file 1: Figure S2f).

In this trial, locomotor activity was significantly higher in *Fmr1* KO mice compared to WT mice [main effect of genotype: $F(1,112) = 5.76$, $p < 0.05$], and this effect was not abolished by BMS-204352 treatment [interaction genotype x treatment: $F(1,112) < 1$, NS; Additional file 1: Figure S2i].

BMS-204352 reverses impaired emotional behavioral phenotype

The emotional component was evaluated by the elevated plus maze. As described in the literature [29,30] *Fmr1* KO mice presented reduced non-social anxiety in this

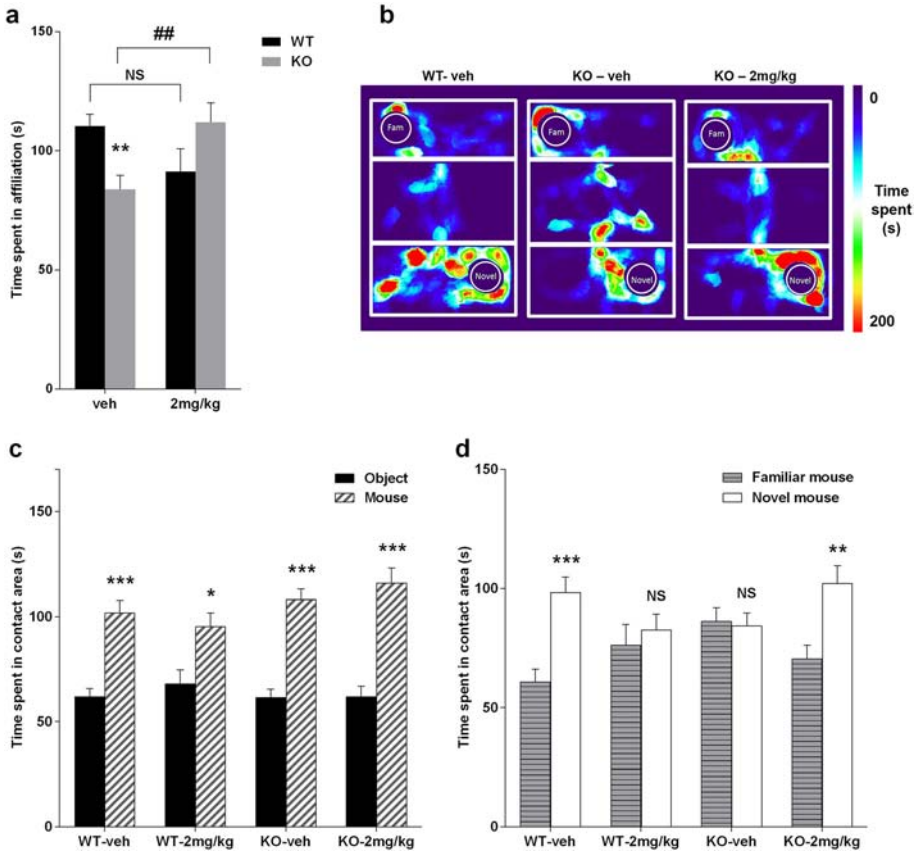

Figure 2 BMS-204352 effects on Social behaviors. Mice were administered with BMS-204352 2 mg/kg or vehicle (veh) and subjected to social behavioral tests 30 min after the injection. **(a)** Histograms represent time spent in affiliative behaviors obtained in two laboratories. A three-way ANOVA indicated a non-significant effect of laboratory, but a significant interaction between genotype and treatment. Vehicle-treated *Fmr1* KO mice spent less time in affiliative behavior compared to WT, but BMS-204352 injection restored a normal social investigation. (*n*: WT-veh = 27; WT-2 mg/kg = 16; KO-veh = 25; KO-2 mg/kg = 17). NS, not significant; **p < 0.05 compared to the corresponding WT; $^{\#\#}$p < 0.05 compared the corresponding treated group. **(b)** Illustration of BMS-204352 effect on the three-chamber test by a pseudo-colored heat map representing time spent at each positions related to the social preference trial (fam: familiar mouse, novel: novel mouse). **(c)** Preference for a conspecific *versus* an object in the three-chamber test was measured by the time spent in the contact area when a stranger mouse and an object were accessible. Repeat-measures ANOVA indicated that vehicle-treated KO mice like WT shown a preference for the mouse *versus* the object, and that BMS-204352 treatment had no effect. **(d)** Preference for a novel *versus* a familiar mouse, was measured by the time spent in the contact area when a stranger and a familiar mouse were accessible. In vehicle-treated groups a preference for the novel mouse was only observed in WT. This preference was rescued by BMS-204352 treatment in *Fmr1* KO mice. (*n*: WT-veh = 34; WT-2 mg/kg = 19; KO-veh = 42; KO-2 mg/kg = 25). NS, not significant; *p < 0.05, **p < 0.01 and ***p < 0.001 (object *versus* mouse, familiar *versus* novel mouse). Data represent mean ± s.e.m.

maze. Anxious mice naturally avoid open spaces and spend more time in closed arms, and less anxious mice spend more time exploring the open arms. The percentage of time spent in open arms is usually used as anxiety measure. The analysis demonstrated that *Fmr1* KO mice presented an elevated percentage compared to WT mice [$F(1,77) = 8.51$, $p < 0.001$; Figure 3a]. Also, they presented higher number of entries and time spent in open arms than WT mice [number of entries: $F(1,77) = 4.04$, $p < 0.05$; time spent: $F(1,77) = 9.38$, $p < 0.01$] (Additional file 1: Figure S3c and S3a, respectively), but the treatment had no effect [$F(1,77) < 1$, NS]. On the other hand, the time spent in closed arms did not differ between groups [$F(1,77) < 1$, NS; Additional file 1: Figure S3b]. Also, the amount of time spent in the extremities of the open arms has been established as a good index of anxiety [31-34]. *Fmr1* KO mice spent more time in the extremity of open arms [genotype effect: $F(1,77) = 5.31$, $p < 0.05$] and this was corrected by BMS-204352 that also increased non-social anxiety in WT mice [treatment effect: $F(1,77) = 4.06$, $p < 0.05$; Figure 3b].

For the distance moved, *Fmr1* KO mice were hyperactive compared to WT mice, and this genotype difference disappeared following BMS-204352 treatment [interaction genotype × treatment: $F(1,77) = 8.08$, $p < 0.01$]. Indeed, BMS-204352 significantly increased locomotion in WT mice, while tended to decrease it in *Fmr1* KO mice (Additional file 1: Figure S3d).

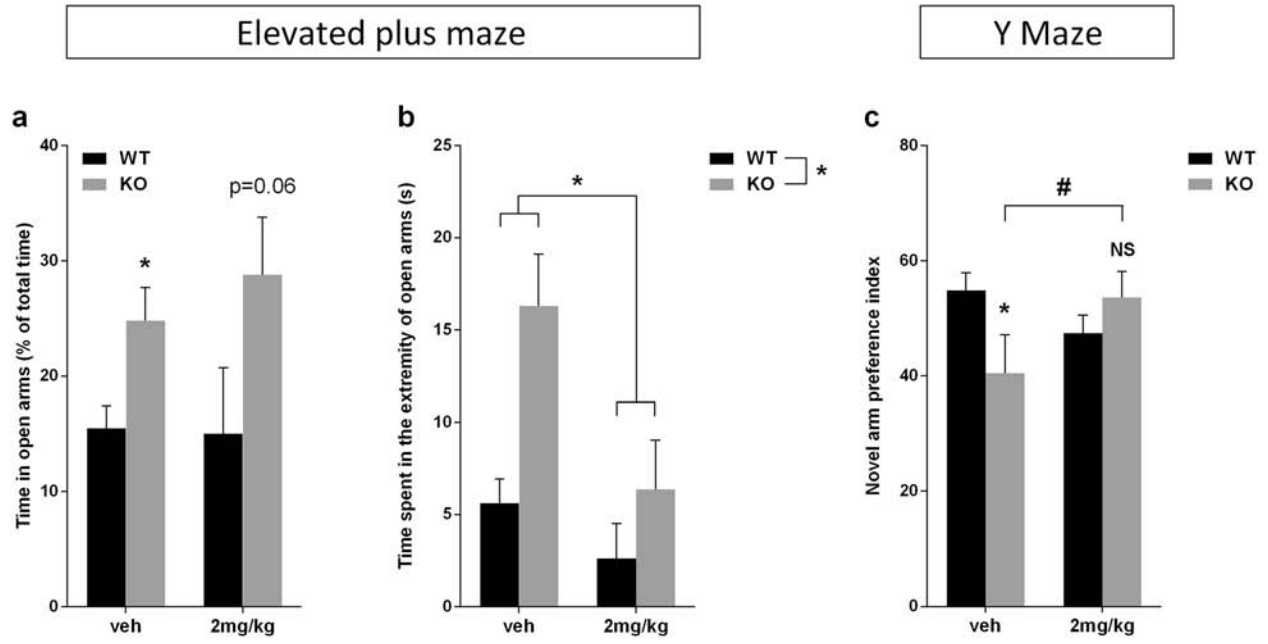

Figure 3 BMS-204352 effects on Emotionality and Spatial memory. (a) Percentage of time spent in open arms time in the elevated plus maze was calculated as the time in open arms/all arms x100. Analysis indicated a main effect of genotype, but no difference between genotypes and treatments was observed. **(b)** Time spent in the extremity of open arms was also evaluated and showed that *Fmr1* KO mice exhibited reduced non-social anxiety in comparison to WT mice. This was corrected by BMS-204352 treatment that increased anxiety in both genotypes. *$p < 0.05$ (*n*: WT-veh = 32; WT-2 mg/kg = 7; KO-veh = 32; KO-2 mg/kg = 10). **(c)** Preference index for a novel arm in the Y-maze was determined by the time spent in the novel arm/ time spent in all 3 arms. *Fmr1* KO mice presented spatial memory impairments through their incapacity to distinguish between novel and familiar arms. BMS-204352 treatment restored this cognitive defect to WT level. Significance was determined using two-way ANOVA with *post-hoc* PLSD test. NS, not significant; *$p < 0.05$, *vs.* WT; $^{\#}p < 0.05$ *vs.* KO-veh (*n*: WT-veh = 7; WT-2 mg/kg = 6; KO-veh = 7; KO-2 mg/kg = 7). Data represent mean ± s.e.m.

BMS-204532 reverses impaired cognitive behavioral phenotype

The cognitive component was investigated through a hippocampus-dependent test, the Y maze for spontaneous alternation [35]. A two-trial memory task in a Y-maze, based on a free-choice exploration paradigm, has been previously developed to study recognition processes [36,37]. *Fmr1* KO mice showed a deficit in spontaneous alternation and it was abolished by BMS-204352 treatment. The analysis of the percent novelty preference index demonstrated that spontaneous alternation was reduced in vehicle-treated *Fmr1* KO mice *versus* vehicle-treated WT mice, while returned to WT levels in BMS-treated *Fmr1* KO group, [interaction genotype x treatment: $F(1,23) = 4.78$, $p < 0.05$; *post-hoc*: $p = 0.048$; Figure 3c]. Moreover, the distance moved during the test did not differ between experimental groups: there was no effect of genotype or treatment [$F(1,23) = 2.52$, $p = 0.13$; $F(1,23) = 1.17$, $p = 0.29$ respectively] (Additional file 1: Figure S3e).

BMS-204352 impacts brain metabolites level in vivo

It has been suggested that glutamate signaling cascade in the absence of FRMP played a causal role in the behavioral phenotype of *Fmr1* KO mice [38]. *In vivo* effects of 2 mg/kg BMS-204352 treatment on glutamate concentration were studied by MRS experiments on the hippocampus structure. Vehicle-treated *Fmr1* KO mice presented a significant lower ($p = 0.009$) hippocampal glutamate concentration compared to vehicle-treated WT mice. Injection of 2 mg/kg BMS-204352 in *Fmr1* KO mice led to a significant ($p = 0.043$) increase of glutamate level (Figure 4a). Interestingly, this injection restored a WT glutamate level since no difference was observed between 2 mg/kg BMS-204352-treated *Fmr1* KO and vehicle-treated WT mice. An increased in glutamate was also observed ($p = 0.043$) after BMS-204352 injection in WT mice.

BMS-204352 single injection also restored the concentration of myo-inositol. Indeed, myo-inositol level was slightly higher ($p = 0.053$) with an important dispersion in vehicle-treated *Fmr1* KO mice compared to vehicle-treated WT littermates (Figure 4b). In *Fmr1* KO mice BMS-204352 induced a significant decrease ($p = 0.002$) in myo-inositol concentration, reaching a concentration similar to vehicle-treated WT level. A similar decrease ($p = 0.038$) was observed after BMS-204352 treatment in

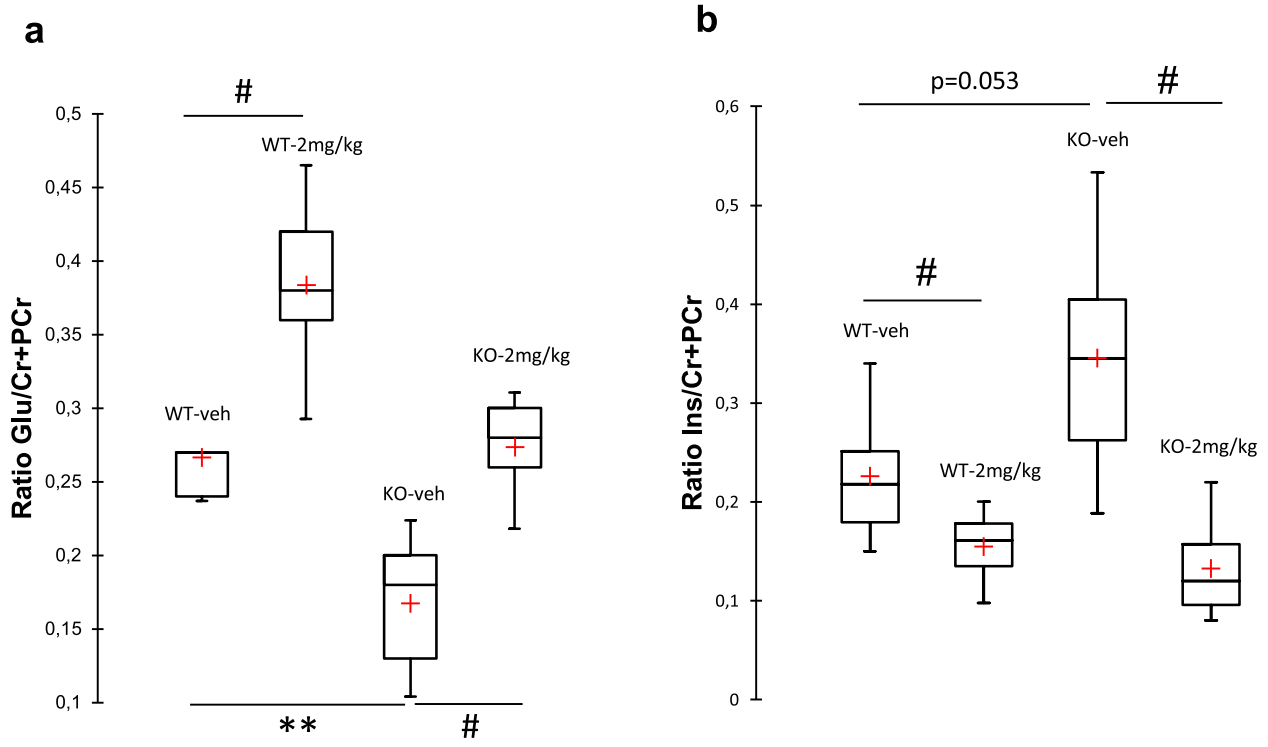

Figure 4 BMS-204352 effects on glutamate and myo-inositol brain level. Mice (3–5 months) were administered with BMS-204352 at 2 mg/kg or vehicle (veh) and subjected to MRS test 30 min after the injection. Box plots (shown as the minimum, 1st quartile, mean, median, 2nd quartile and maximum values) represent **(a)** glutamate and **(b)** myo-inositol concentrations in arbitrary unit (AU), after normalization by Creatine/Phosphocreatine, in vehicle or BMS-204352-treated-WT and *Fmr1* KO mice. Glutamate level was significantly decreased in *Fmr1* KO mice compared to WT, whereas myo-inositol concentration was increased. Furthermore, a single injection of BMS-204352 rescued a WT like metabolites concentration. Significance between WT and KO was determined using Mann–Whitney test and impact of treatment was determined by Wilcoxon test. NS, not significant; **$p < 0.01$ for genotype comparison; $^{\#}p < 0.05$ for treatment comparison (*n*: WT-veh = 10; WT-2 mg/kg = 10; KO-veh = 10; KO-2 mg/kg = 10).

WT mice. Vehicle-treated *Fmr1* KO mice also presented significant deregulation of Taurine, N-Acetyl-Aspartate and Lactate metabolites (Additional file 1: Figure S4). In those mice, BMS-204352 (2 mg/kg) treatment had a significant effect on taurine and lactate concentration, restoring a WT level.

Discussion

Currently, accumulated evidences highlight BKCa channel as a potential therapeutic target for FXS. Indeed, *in vitro* in the absence of FMRP, BKCa alpha subunit protein (KCNMA1) expression is decreased both in primary neuron cultures of *Fmr1* KO mice [20] and lymphoblastoid cells derived from FXS patients (Additional file 1: Figure S5). Moreover, Deng *et al.* [3] demonstrated that BKCa currents were decreased in CA3 pyramidal neuron of *Fmr1* KO mice. Dysregulation of neurotransmitter release and short-term plasticity, as a consequence of these defects, should in turn contributed to the behavior and cognitive deficits [3]. Therefore, we hypothesized that enhancing BKCa channel activity could rescue FXS neurobehavioral phenotypes. To this end, we chose to test the effects of fluoro-oxindole BMS-204352, a sensitive BKCa channel opener [17,39], on the dendritic spines phenotype, behavioral impairments and the cellular hippocampal metabolism of the murine FXS model, the *Fmr1* KO mice.

At first, before conducting our *in vivo* experiments, we decided to evaluate BMS-204352 *in vitro* effects on dendritic spine morphology in primary neuron cell cultures from *Fmr1* KO embryos [4,6,40-42]. We showed that an acute treatment with BMS-204352 was able to restore the dendritic defects, typically observed in *Fmr1* neurons, to a regular level. This finding suggests that BKCa channel, which was previously defined as a determinant of presynaptic activity [15], should contribute to synaptogenesis. Hypothetically, these neuromorphological changes observed should directly link to local modulation of potassium flux, induced by an open conformational state of BK channel, as other potassium channels have been demonstrated to be closely associated with filopodia growth, dendritic development and/or neuronal differentiation [43,44]. However, the link between local potassium ionic flux and the cellular pathway involved in dendritic maturation is worth being further explored.

The *in vitro* beneficial effects of several molecules on dendritic spine maturation of *Fmr1* KO mice were correlated with *in vivo* endophenotype observations [6,45,46]. BMS-204352 effects were investigated *in vivo* after intraperitoneal injection at 2 mg/kg [23,24]. As previously described, adult *Fmr1* KO mice exhibited social behavioral impairments in a direct social interaction test as demonstrated by lower level of affiliative behaviors [27,28,47]. After injection of BMS-204352 2 mg/kg, affiliative behaviors in *Fmr1* KO mice were significantly increased to the WT level. To confirm and complete the results obtained in the direct social interaction test, we performed a three-chamber test, which showed social impairments in *Fmr1* KO mice demonstrated by their inability to distinguish a novel mouse from a familiar one, as previously described [27,28,47]. The observed effect in sociability components is very robust, as these tests were performed by different experimenter, in two different laboratories with two distinct cohorts of mice. Interestingly, during trial 2 (sociability trial), *Fmr1* KO mice and their WT littermates exhibited the same level of interest in the stimulus mouse compared to a non-social stimulus [27,28,47]. During this trial only olfactory and visual modalities come into play, and only the experimental mouse can initiate social contact. The reduced level of interactions of *Fmr1* KO mice during the direct social interaction test, is therefore most probably due to social anxiety induced by the direct contact with the stimulus mouse. In contrast, *Fmr1* KO mice exhibited reduced non-social anxiety compared to WT mice in the elevated plus maze [29]. Reduced non-social anxiety was rescued to normal levels after BMS-204352 treatment based on the time spent in the extremity of open arms. An acute treatment with BMS-204352 was therefore able to rescue the social deficits of *Fmr1* KO mice by increasing affiliative behaviors, decreasing social anxiety and enhancing social recognition. Our results are extended by spatial recognition test using Y-maze test. *Fmr1* KO mice presented spatial memory impairments through their incapacity to distinguish novel arm from familiar arms. This is in agreement with several studies which use different maze [6,8,48]. BMS-204352 treatment restored this cognitive impairment to WT level.

Therefore, we demonstrated that an acute BMS-204352 treatment at 2 mg/kg restored a normal phenotype in social, cognitive and emotional components by improving sociability, social and spatial recognition, and social/non-social anxiety.

Currently, distinct therapeutic targets are under investigations involving *Fmr1* KO mice. Many studies provide compelling evidence for several potential efficient pharmacological products. However, the comparison between those studies and ours was limited because they involved different mazes, molecules or strains. To our knowledge, our study is the first investigation to evaluate the efficiency of an acute treatment on the three main components of adult *Fmr1* knock-out mouse behavioral deficits: social, emotional and cognitive. Moreover, long-term study will provide additional data on phenotypic improvements already observed with a single-dose treatment. Indeed, positive behavioral effect due to BMS-20352 single injection were no longer observed 180 minutes after the injection (Additional file 1: Figure S6), likely due to the short BMS-204352 half-life (Additional file 1: Figure S1). Therefore, this long-term investigation will allow us to explore further cognitive traits such as learning and long-term memory, since Typlt *et al.* proposed a crucial role of the BKCa channels in learning [19].

As suggested before, in the absence of FMRP, numerous impairments of synaptic function were described with direct consequences on signaling cascades and cellular metabolism [3,49,50]. Moreover, presynaptic location of BKCa channels might provide a homeostatic mechanism for regulating synaptic transmission [3,15]. Accordingly, we investigated BMS-204352 treatment effects on metabolic profile of *Fmr1* KO mice hippocampus with MRS method. The advantage of this non-invasive method was to obtain a metabolomic profile of a brain area in anaesthetized animals, unlike to other methods which required dissected tissues. By this MRS method, we showed that glutamate concentration was significantly reduced in our adult *Fmr1* KO mice compared to WT mice. A similar glutamate decrease was also described by ^{1}H HR-MAS NMR spectroscopy on cortex of 12 days of age *Fmr1* KO FVB mice, suggesting that excitatory input appears compromised [50,51]. Our result support the hypothesis previously published that glutamatergic function disturbance might contribute to FXS phenotypes in mice [3,49,50]. In our conditions, this glutamate reduction was rescued by a single injection of BMS-204352 at 2 mg/kg. Consistently with our behavioral experiments, BKCa channel opener should be a valuable tool to regulate glutamate neurotransmission and metabolism.

In addition, myo-inositol, mainly presents in astrocytes [52], was found deregulated in *Fmr1* KO mice. Myo-inositol concentration is altered in brain disorders such as Alzheimer's disease and often, elevation of myo-inositol level reflects an astrocytic activation [53]. In our study, *Fmr1* KO mice also presented an elevated myo-inositol concentration suggesting an activation of astrocytes. This was consistent with glial fibrillary acidic protein (GFAP) and its mRNA up-expressions previously observed in several brain regions [51]. All of these data are consistent with an astrocyte involvement in synaptic defects observed in *Fmr1* KO mice [54-56]. Interestingly, BMS-204352 acute treatment decreased the myo-inositol concentration to WT level, however molecular and cellular pathways involved remained unclear. Based on

these results, we suggest that BMS-204352 should be able to treat functional alteration of the tripartite synapse involved in the Fmr1 phenotype [55,56].

Conclusion

All these data reinforced evidences that BKCa pathway has to be explored as a new interesting therapeutic target for FXS patient. Therefore, our results suggested that BKCa channel opener molecule (BMS-204352) constitutes a promising potential medication for FXS patients correcting a broad spectrum of behavioral impairments (social, emotional and cognitive). BMS-204352 went up to phase III trial for the treatment of acute ischemic stroke but failed to show improvement against placebo. However during trials no organ toxicity or adverse effects were found [24]. This allows us to look forward with confidence to clinical trials involving few FXS patients.

Additional file

Additional file 1: Additional Methods description and Results are presented in Additional file 1.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

BH, SP, JP, AM, OP and SB conceived and designed the experiments; BH, SP, AL and OP performed the behavioral experiments; SM and BH performed the SRMN; AQ, AM, ND and OP performed the *in vitro* experiments; DC produced deuterated molecule for kinetic assay; SL and LG performed molecule kinetic assay; BH, SP and OP analyzed the data; BH, AM and OP wrote the manuscript; SP, BL, ND, FL, WC, JP, AM, and SB coordinated and helped to draft the manuscript. All authors read and approved the final manuscript.

Authors' information

Betty Hebert and Susanna Pietropaolo are equal first contributors (co-first authors). Olivier Perche and Sylvain Briault are equal contributors (co-final authors).

Acknowledgments

We would like to thank Mélanie Marcos for her excellent technical support and Alexandre Herpin, Jerôme Larrigaldie and Ludovic Mercier for animal breeding. We also thank Jean-Claude Beloeil and Valérie Quesniaux for their continued support of the project. Research was supported in part by grants from Fondation de France 015448, The FRAXA Research Foundation (USA), FEDER Autism 35106, Fondation Lejeune, CNRS (Soutien au transfert 04388–02), Région Centre, Regional Hospital of Orléans, the University of Orléans and Project FP7 GENCODYS (n°241995, to FL). W.C. and S.P. were supported by grants from the March of Dimes (12-FY05-1198), Conseil Régional d'Aquitaine, CNRS, and the University of Bordeaux 1 to W.C.

Author details

[1]UMR7355, CNRS, Orléans, France. [2]Experimental and Molecular Immunology and Neurogenetics, University of Orléans, 3b rue de la Férollerie, 45071 Orléans, Cedex 2, France. [3]The Aquitaine Institute for Cognitive and Integrative Neuroscience, UMR 5287, CNRS, Talence, France. [4]The Aquitaine Institute for Cognitive and Integrative Neuroscience, University of Bordeaux, Avenue des Facultés, 33405 Talence, France. [5]Centre de Biophysique Moléculaire, UPR4301, CNRS, University of Orléans, Rue Charles Sadron, 45071 Orléans, Cedex, France. [6]Genetic department, Regional Hospital, 14 Avenue de l'Hôpital, 45100 Orléans, France. [7]UMR CNRS 6014 C.O.B.R.A., INSA of Rouen, 1 rue Tesnière, 76821 Mont Saint Aignan, France. [8]INSERM, U930, Tours 37032, France. [9]UMR Imagerie et cerveau, François-Rabelais University, Tours 37000, France.

Received: 10 February 2014 Accepted: 21 July 2014
Published: 1 August 2014

References

1. Hagerman RJ: **Advances in the treatment of fragile X syndrome.** *Pediatrics* 2009, **123:**378–390.
2. Darnell JC, Van Driesche SJ, Zhang C, Hung KY, Mele A, Fraser CE, Stone EF, Chen C, Fak JJ, Chi SW, Licatalosi DD, Richter JD, Darnell RB: **FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism.** *Cell* 2011, **146:**247–261.
3. Deng PY, Rotman Z, Blundon JA, Cho Y, Cui J, Cavalli V, Zakharenko SS, Klyachko VA: **FMRP regulates neurotransmitter release and synaptic information transmission by modulating action potential duration via BK channels.** *Neuron* 2013, **77:**696–711.
4. Irwin SA, Galvez R, Greenough WT: **Dendritic spine structural anomalies in fragile-X mental retardation syndrome.** *Cereb Cortex* 2000, **10:**1038–1044.
5. Bakker CE, Verheij C, Willemsen R, Helm R, Oerlemans F, Vermey M: **Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium.** *Cell* 1994, **78:**23–33.
6. Bilousova TV, Dansie L, Ngo M, Aye J, Charles JR, Ethell DW, Ethell IM: **Minocycline promotes dendritic spine maturation and improves behavioural performance in the fragile X mouse model.** *J Med Genet* 2009, **46:**94.
7. Mineur YS, Sluyter F, de Wit S, Oostra BA, Crusio WE: **Behavioral and neuroanatomical characterization of the Fmr1 knockout mouse.** *Hippocampus* 2002, **12:**39–46.
8. 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:**e17073.
9. Gross C, Berry-Kravis EM, Bassell GJ: **Therapeutic strategies in fragile X syndrome: dysregulated mGluR signaling and beyond.** *Neuropsychopharmacology* 2012, **37:**178–195.
10. Yan QJ, Rammal M, Tranfaglia M, Bauchwitz RP: **Suppression of two major fragile X syndrome mouse model phenotypes by the mGluR5 antagonist MPEP.** *Neuropharmacology* 2005, **49:**1053–1066.
11. Yuskaitis CJ, Mines MA, King MK, Sweatt JD, Miller CA, Jope RS: **Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of fragile X syndrome.** *Biochem Pharmacol* 2010, **79:**632–646.
12. Pacey LK, Tharmalingam S, Hampson DR: **Subchronic administration and combination metabotropic glutamate and GABAB receptor drug therapy in fragile X syndrome.** *J Pharmacol Exp Ther* 2011, **338:**897–905.
13. Lee HY, Jan LY: **Fragile X syndrome: mechanistic insights and therapeutic avenues regarding the role of potassium channels.** *Curr Opin Neurobiol* 2012, **22:**887–894.
14. Latorre R, Brauchi S: **Large conductance Ca2 + −activated K + (BK) channel: activation by Ca2+ and voltage.** *Biol Res* 2006, **39:**385–401.
15. Salkoff L, Butler A, Ferreira G, Santi C, Wei A: **High-conductance potassium channels of the SLO family.** *Nat Rev Neurosci* 2006, **7:**921–931.
16. Nardi A, Olesen SP: **BK channel modulators: a comprehensive overview.** *Curr Med Chem* 2008, **15:**1126–1146.
17. Laumonnier F, Roger S, Guerin P, Molinari F, M'Rad R, Cahard D, Belhadj A, Halayem M, Persico AM, Elia M, Romano V, Holbert S, Andres C, Chaabouni H, Colleaux L, Constant J, Le Guennec JY, Briault S: **Association of a functional deficit of the BKCa channel, a synaptic regulator of neuronal excitability, with autism and mental retardation.** *Am J Psychiatry* 2006, **163:**1622–1629.
18. Higgins JJ, Hao J, Kosofsky BE, Rajadhyaksha AM: **Dysregulation of large-conductance Ca2 + −activated K + channel expression in nonsyndromal mental retardation due to a cereblon p.R419X mutation.** *Neurogenetics* 2008, **9:**219–223.
19. Typlt M, Mirkowski M, Azzopardi E, Ruettiger L, Ruth P, Schmid S: **Mice with deficient BK channel function show impaired prepulse inhibition and spatial learning, but normal working and spatial reference memory.** *PLoS One* 2013, **8:**e81270.
20. Liao L, Park SK, Xu T, Vanderklish P, Yates JR 3rd: **Quantitative proteomic analysis of primary neurons reveals diverse changes in synaptic protein content in fmr1 knockout mice.** *Proc Natl Acad Sci U S A* 2008, **105:**15281–15286.
21. Briault S, Perche O: **Compositions for the treatment of fragile X syndrome**. In *Book Compositions for the Treatment of Fragile X Syndrome*, Editor ed. ^eds. City: WO Patent 2/013/001/412; 2013.

22. Ethell IM, Irie F, Kalo MS, Couchman JR, Pasquale EB, Yamaguchi Y: **EphB/syndecan-2 signaling in dendritic spine morphogenesis.** *Neuron* 2001, **31:**1001–1013.
23. Gribkoff VK, Starrett JE Jr, Dworetzky SI: **Maxi-K potassium channels: form, function, and modulation of a class of endogenous regulators of intracellular calcium.** *Neuroscientist* 2001, **7:**166–177.
24. Jensen BS: **BMS-204352: a potassium channel opener developed for the treatment of stroke.** *CNS Drug Rev* 2002, **8:**353–360.
25. Meme S, Calas AG, Montecot C, Richard O, Gautier H, Gefflaut T, Doan BT, Meme W, Pichon J, Beloeil JC: **MRI characterization of structural mouse brain changes in response to chronic exposure to the glufosinate ammonium herbicide.** *Toxicol Sci* 2009, **111:**321–330.
26. Galvez R, Greenough WT: **Sequence of abnormal dendritic spine development in primary somatosensory cortex of a mouse model of the fragile X mental retardation syndrome.** *Am J Med Genet A* 2005, **135:**155–160.
27. McNaughton CH: **Evidence for social anxiety and impaired social cognition in a mouse model of fragile X syndrome.** *Behav Neurosci* 2008, **122:**293–300.
28. Moy SS, Nadler JJ, Young NB, Nonneman RJ, Grossman AW, Murphy DL, D'Ercole AJ, Crawley JN, Magnuson TR, Lauder JM: **Social approach in genetically engineered mouse lines relevant to autism.** *Genes Brain Behav* 2009, **8:**129–142.
29. Liu Z-H, Smith CB: **Dissociation of social and nonsocial anxiety in a mouse model of fragile X syndrome.** *Neurosci Lett* 2009, **454:**62–66.
30. Qin M, Xia Z, Huang T, Smith CB: **Effects of chronic immobilization stress on anxiety-like behavior and basolateral amygdala morphology in Fmr1 knockout mice.** *Neuroscience* 2011, **194:**282–290.
31. Cardenas F, Lamprea MR, Morato S: **Vibrissal sense is not the main sensory modality in rat exploratory behavior in the elevated plus-maze.** *Behav Brain Res* 2001, **122:**169–174.
32. Estanislau C, Morato S: **Behavior ontogeny in the elevated plus-maze: prenatal stress effects.** *Int J Dev Neurosci* 2006, **24:**255–262.
33. Garcia AM, Martinez R, Brandao ML, Morato S: **Effects of apomorphine on rat behavior in the elevated plus-maze.** *Physiol Behav* 2005, **85:**440–447.
34. Martinez RC, Garcia AM, Lamprea MR, Morato S: **Thermal stress decreases general motor activity of rats in the elevated plus-maze but does not alter aversion to the open arms.** *Behav Brain Res* 2007, **182:**135–139.
35. Dellu F, Contarino A, Simon H, Koob G, Gold L: **Genetic differences in response to novelty and spatial memory using a two-trial recognition task in mice.** *Neurobiol Learn Mem* 2000, **73:**31–48.
36. Dellu F, Fauchey V, Moal ML, Simon H: **Extension of a new two-trial memory task in the rat: influence of environmental context on recognition processes.** *Neurobiol Learn Mem* 1997, **67:**112–120.
37. Dellu F, Mayo W, Cherkaoui J, Le Moal M, Simon H: **A two-trial memory task with automated recording: study in young and aged rats.** *Brain Res* 1992, **588:**132–139.
38. Bear MF, Huber KM, Warren ST: **The mGluR theory of fragile X mental retardation.** *Trends Neurosci* 2004, **27:**370–377.
39. Gribkoff VK, Starrett JE, Dworetzky SI, Hewawasam P, Boissard CG, Cook DA, Frantz SW, Heman K, Hibbard JR, Huston K: **Targeting acute ischemic stroke with a calcium-sensitive opener of maxi-K potassium channels.** *Nat Med* 2001, **7:**471–477.
40. Thomas CC, Combe CL, Dyar KA, Inglis FM: **Modest alterations in patterns of motor neuron dendrite morphology in the Fmr1 knockout mouse model for fragile X.** *Int J Dev Neurosci* 2008, **26:**805–811.
41. Grossman AW, Aldridge GM, Lee KJ, Zeman MK, Jun CS, Azam HS, Arii T, Imoto K, Greenough WT, Rhyu IJ: **Developmental characteristics of dendritic spines in the dentate gyrus of Fmr1 knockout mice.** *Brain Res* 2010, **1355:**221–227.
42. Wei H, Dobkin C, Sheikh AM, Malik M, Brown WT, Li X: **The therapeutic effect of memantine through the stimulation of synapse formation and dendritic spine maturation in autism and fragile X syndrome.** *PLoS One* 2012, **7:**e36981.
43. Zhou X, Song M, Chen D, Wei L, Yu SP: **Potential role of KCNQ/M-channels in regulating neuronal differentiation in mouse hippocampal and embryonic stem cell-derived neuronal cultures.** *Exp Neurol* 2011, **229:**471–483.
44. Liebau S, Vaida B, Proepper C, Grissmer S, Storch A, Boeckers TM, Dietl P, Wittekindt OH: **Formation of cellular projections in neural progenitor cells depends on SK3 channel activity.** *J Neurochem* 2007, **101:**1338–1350.
45. Boda B, Mendez P, Boury-Jamot B, Magara F, Muller D: **Reversal of activity-mediated spine dynamics and learning impairment in a mouse model of Fragile X syndrome.** *Eur J Neurosci* 2014, **39:**1130–1137.
46. De Vrij F, Levenga J, Van der Linde HC, Koekkoek SK, De Zeeuw CI, Nelson DL, Oostra BA, Willemsen R: **Rescue of behavioral phenotype and neuronal protrusion morphology in Fmr1 KO mice.** *Neurobiol Dis* 2008, **31:**127–132.
47. Mines MA, Yuskaitis CJ, King MK, Beurel E, Jope RS: **GSK3 influences social preference and anxiety-related behaviors during social interaction in a mouse model of fragile X syndrome and autism.** *PLoS One* 2010, **5:**e9706.
48. Sun MK, Hongpaisan J, Lim CS, Alkon DL: **Bryostatin-1 restores hippocampal synapses and spatial learning and memory in adult fragile x mice.** *J Pharmacol Exp Ther* 2014, **349:**393–401.
49. Shi D, Xu S, Waddell J, Scafidi S, Roys S, Gullapalli RP, McKenna MC: **Longitudinal in vivo developmental changes of metabolites in the hippocampus of Fmr1 knockout mice.** *J Neurochem* 2012, **123:**971–981.
50. Davidovic L, Navratil V, Bonaccorso CM, Catania MV, Bardoni B, Dumas ME: **A metabolomic and systems biology perspective on the brain of the fragile X syndrome mouse model.** *Genome Res* 2011, **21:**2190–2202.
51. Yuskaitis CJ, Beurel E, Jope RS: **Evidence of reactive astrocytes but not peripheral immune system activation in a mouse model of Fragile X syndrome.** *Biochimica et Biophysica Acta (BBA)-Mol Basis Dis* 2010, **1802:**1006–1012.
52. Brand A, Richter-Landsberg C, Leibfritz D: **Multinuclear NMR studies on the energy metabolism of glial and neuronal cells.** *Dev Neurosci* 1993, **15:**289–298.
53. Miller BL, Moats RA, Shonk T, Ernst T, Woolley S, Ross BD: **Alzheimer disease: depiction of increased cerebral myo-inositol with proton MR spectroscopy.** *Radiology* 1993, **187:**433–437.
54. Yang Q, Feng B, Zhang K, Guo Y-y, Liu S-b, Wu Y-m, Li X-q, Zhao M-g: **Excessive astrocyte-derived neurotrophin-3 contributes to the abnormal neuronal dendritic development in a mouse model of fragile x syndrome.** *PLoS Genet* 2012, **8:**e1003172.
55. Higashimori H, Morel L, Huth J, Lindemann L, Dulla C, Taylor A, Freeman M, Yang Y: **Astroglial FMRP-dependent translational down-regulation of mGluR5 underlies glutamate transporter GLT1 dysregulation in the fragile X mouse.** *Hum Mol Genet* 2013, **22:**2041–2054.
56. Jacobs S, Doering LC: **Astrocytes prevent abnormal neuronal development in the fragile x mouse.** *J Neurosci* 2010, **30:**4508–4514.

doi:10.1186/s13023-014-0124-6
Cite this article as: Hébert *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.

Submit your next manuscript to BioMed Central and take full advantage of:

- **Convenient online submission**
- **Thorough peer review**
- **No space constraints or color figure charges**
- **Immediate publication on acceptance**
- **Inclusion in PubMed, CAS, Scopus and Google Scholar**
- **Research which is freely available for redistribution**

Submit your manuscript at
www.biomedcentral.com/submit

BioMed Central

Additional file 1 / BRIAULT et al_2014

Supplementary Methods

Plasma and Brain sampling.

Blood (cardiac puncture) and brain samples from mice were obtained prior to dosing and postdose (2mg/Kg, bolus) at 5, 30, 60, 90, 120, 180, 240 and 360 min (n=6 per time). Blood samples were drawn into Vacutainer® test tubes with Li-heparin. The samples were centrifuged at 2000 g for 10 minutes at 4 ºC, and plasma was separated and stored in Eppendorf tubes at -20º C until analysis. Each brain were carefully rinsed with saline solution and weighted individually. After homogenisation with 1mL of saline solution (NaCl 0.9%) for 400mg of brain, samples were frozen at -20º C until analysis.

BMS assays: plasma and brain samples.

BMS was quantified in plasma and brain samples using a sensitive and selective LC-MS/MS method. Briefly, to 50µL of plasma or brain samples were added the internal standard BMS – D3 (IS) and 100µL of acetonitrile. After centrifugation, 10µL of the supernatant was directly injected into the LC-MS/MS system equipped with an ion electrospray interface in negative mode. (5500 QTRAP, ABsciex, Saint Quentin en Yvelines, France) and an ultra high-performance liquid chromatography (Shimadzu, Saint Quentin en Yvelines, France). Data were acquired in multiple reaction monitoring including: m/z 358.9 → m/z 339 and m/z 358.9 → m/z 324 for BMS and m/z 360.9 → m/z 340.9 for the IS. The chromatographic separation was performed on a C18 BEH (50mm x 2.1mm; 1.7µm) column (Waters, Saint Quentin en Yvelines, France) maintained at 35°C. The mobile phase was composed of a mixture of solvent A (water, formic acid (99.9/0.1, v/v)) and solvent B (acetonitrile, formic acid (99.9/0.1, v/v)), delivered at a flow rate of 0.35 ml/min in the following gradient mode: 20%

to 100% of B from 0 to 1min, followed by linear gradient to 100% of B from 1 to 2.8 min. The column was equilibrated from 2.9 to 4.5 min with 20% of B. The retention time of BMS was 2.8 min. Calibration curves were linear from 0.5 µg/ml (limit of quantification) to 50 ng/ml range. Within and between-day accuracy and precision at three levels: (0.5; 7; 30 ng/ml) were within acceptable limits (<15%).

Animals

All experiments were done with adult (3-5 months old) male *Fmr1* KO and their wild-type (WT) littermates of C57BL/6J background. *Fmr1* mice were obtained from the colony of the Aquitaine Institute for Cognitive and Integrative Neuroscience in Bordeaux (France). Breeding trios were formed by mating two heterozygous females with a WT male (purchased from Janvier, Le Genest St Isle). After 2 weeks the sire was removed, the females were single caged and left undisturbed until weaning of the pups. Mice were weaned at 21 days of age and group-housed with their same-sex littermates. On the same day, tail samples were collected for DNA extraction and subsequent PCR assessment of genotypes as previously described (Dutch-Belgian Fragile X Consortium 1994). All animals were housed in polycarbonate standard cages (26.7 x 20.7 x 14 cm in size; Tecniplast, Limonest, France), provided with sawdust bedding (SAFE, Augy, France), and a stainless steel wire lid. Food chow (SAFE, Augy, France) and water were provided *ad libitum*. Only male mice were used for the present studies; they were maintained in a male colony room under temperature (22°C) and humidity-controlled (55%) conditions with a 12:12 hr light–dark cycle (lights on at 7 a.m.). The present experimental protocol received full review and approval by the regional animal care and use committee (CLE CCO 2011-024) prior to conducting the experiments. All possible efforts were made to reduce the number of animals studied and to avoid their suffering.

Neuronal cell cultures and BMS-204352 treatment

Primary cultures of neurons were prepared from male WT and *Fmr1* KO mice at embryonic day 15 (E15) as previously described [1]. Briefly, the entire brain was dissected out, and single cell suspensions from anterior brain were prepared by mechanical dissociation. Cells were washed in HBSS (10mM Hepes) and resuspended in culture medium (Neurobasal, Invitrogen, Fr) supplemented with 25mM glutamine (BioWest, Nuaille, Fr), B27 complement (Invitrogen, Carlsbad, CA), and 2mM penicillin and streptomycin (BioWest, Nuaille, Fr). Cells were seeded into poly-D-lysine-coated (0.5 mg/ml in borate buffer) coverslips in 24 well plates (60 000 cells/well) and incubated at 37°C in a moist chamber under 5% CO_2. At 14 day *in vitro* (DIV) neurons were used for dendritic spine maturation study with or without BMS-204352 acute treatment (4 hr). BMS-204352 was diluted with DMSO 0.1% in supplemented Neurobasal to obtain 5 µM or 10µM in the culture well. After 4 hours of incubation medium was filled out, and neurons were prepared for DiI labeling.

DiI labeling and dendritic spine maturation

Neurons were labeled using a protocol adapted from Hering [2]. Briefly, male WT and *Fmr1* KO neurons at 14 DIV were washed twice in HBSS, fixed in 4% paraformaldehyde for 15 min and incubated with Vybrant-DiI cell-labeling solution (1:200, Invitrogen) for 30 min at 37°C. Then cultures were washed in warmed PBS, incubated in PBS at 4°C for 24–48 hr to allow dye diffusion within membranes, mounted on glass slides with Fluoromount (Southernbiotech, Invitrogen), and then imaged using a Leica DM6000b microscope (Leica Microsystem, Nanterre, Fr). Filopodia spines length and density were measured using ImageJ Software (http://rsbweb.nih.gov/ij/). Six independent experiments were done sprayed over 6 months. Filopodia were counted in 10-12 neurons/wells with 6 wells/embryos. Filopodia were analyzed on 200 µm length of each neuron. The spine value was averaged in each

independent experiment for the statistical test. The n value refers to the number of independent experiments analyzed.

Behavior

The experiments were conducted in two laboratories: The Experimental and Molecular Immunology and Neurogenetics laboratory in Orléans, France (Laboratory A), and The Aquitaine Institute for Cognitive and Integrative Neuroscience in Bordeaux, France (Laboratory B). Mice were bred and maintained in the two laboratories following identical procedures: the first two behavioral tests, i.e., the direct social interaction test and the three compartment test for sociability and social recognition were conducted in parallel in both laboratories. This experimental strategy was adopted in order to assess the robustness and replicability of the results obtained. The last two tests, i.e., the Y maze for spontaneous alternation and the elevated plus maze were carried out in Laboratory B and Laboratory A, respectively. In order to minimize the number of animals studied, the same mice were used during the first three behavioral tests.

In all behavioral assays, excluding the direct social interaction test, the mouse behavior was recorded by an analogic camera (SD5 - WV-CP500/G, Panasonic) and analyzed by a PC-based video tracking software (EthoVision XT, Noldus Technology, The Netherlands), monitoring also the distance moved and the velocity of the subjects.

Drug administration

The effective dose of BMS-204352 was chosen based on previously published data [3, 4]. BMS-204352 (2mg/kg) diluted in the vehicle solution (DMSO 1/80; Tween 80 1/80; 0.9% NaCl) was administered by a 10 ml/kg single intraperitoneal (i.p) injection. Behavioral tests were performed at the maximal BMS-204352 brain concentration, i.e., 30 min after injection

(Details appear in Supplemental Experimental Procedures and Supplemental Data obtained by LC-MS/MS method, Figure S1).

Direct social interaction test

In the first behavioral test, we assessed mouse direct social interaction. As indicated above, this test was performed in two locations, i.e., in both our laboratories A and B. After the BMS-204352 or vehicle intraperitoneal injection, the subject (WT or *Fmr1* KO mice) was left undisturbed for 30 min into an individual polycarbonate cage (33.2 x 15 x 13 cm in size; Tecniplast, Limonest, Fr), provided with sawdust bedding, and a stainless steel wire lid. A stimulus female mouse (NMRI strain, 12 weeks) was then introduced in the cage for 5 min and mouse behavior was recorded by a numerical camera (Handycam, DCR-SR58E, Sony). Time spent in affiliative behaviors (sum of nose sniffing, anogenital sniffing, body sniffing and allo-grooming) was measured from the videos using the Observer XT software (version 10, Noldus Technology, The Netherlands) by a researcher who was blind to the genotype of test mice. Time spent in non-social behaviors, such as rearing, digging, and self-grooming was also computed.

Three-chamber test for sociability and social recognition

In order to evaluate mouse sociability and social recognition, we used the three-chamber test. The apparatus used in this study was similar to the one used by Nadler [5] and was previously used to test *Fmr1* mice [6]. The three-chamber apparatus was a non-transparent plexiglass box (25 x 50 cm) with two transparent partitions that make left, center, and right chambers (25 x 16.7 cm). Each partition had a square opening (5 x 5 cm) in the bottom center. A cylindrical wire cage (8 cm diameter; red pencil cup) was placed into the right and the left chambers. The test consisted of three phases. In the first 5 min session (habituation), the test mouse was placed in the center chamber, and was allowed to freely explore each chamber, with the wire

cages empty. In the next 5 min session (sociability), an unknown juvenile male (DBA/2j strain), was placed in one of the two wire cages, while an inanimate object was introduced in the other wire cage. Testing in Laboratory B used a plastic grey cylinder as an object, while in Laboratory A a grey plastic mouse object was used. In the last 5 min session (social recognition), a second unknown juvenile male (DBA/2j strain) was placed in the wire cage where the object previously was. Thus, the test mouse would now have the choice between a mouse that was considered as familiar and a novel stranger mouse. The three-chamber apparatus and wire cups were cleaned with 70% ethanol then water and wiped with paper towels between each trial. Allocation of the stimuli was counterbalanced within experimental groups. Time spent in each chamber and time spent within a 3 cm radius proximal to each wire cage (contact area) was measured. Individual movement tracks were analyzed by EthoVision XT and modified by ImageJ software to generate heat maps.

Elevated plus maze test

In order to evaluate the anxiety phenotype of mice, we used the elevated plus maze test. This was a plus-shaped maze, 50 cm elevated above the floor, consisting of two closed arms surrounded by 21 cm high black walls and two open arms (6 x 35 cm). Each mouse was placed in the center (6 x 6 cm) of the maze facing one of the closed arms. During the 5-min test period, mouse behavior was recorded, and time spent in closed arms, center, open arms and extremity of arms was measured. In accordance to software parameters, a mouse was considered to be within an arm of the maze when all four paws were within the arm. The maze was cleaned with 70% ethanol then water and wiped with paper towels between each trial.

Y Maze test

We performed a Y maze test modified in order to assay spatial memory, an hippocampus-dependent spatial recognition component [7]. A two-trial memory task in a Y-maze, based on

a free-choice exploration paradigm, has been previously developed to study recognition processes [8, 9]. This included two phases: in the first 5 min session of acquisition, the animal was introduced at the end of the start arm and allowed to freely explore the two open arms of a Y maze, the third (novel) was blocked by a grey plastic door. The mouse was removed from the apparatus for a 10 min period (inter-trial interval), and left undisturbed in a waiting cage. Then the door was removed, and the mouse was reintroduced in the start arm and allowed to freely explore the entire maze for 2 min (retrieval). For both trials, the testing session started once the animal had left the start arm and first visited the center of the maze. The three arms of the Y maze were identical and spaced at 120° from each other. The Y maze was located in a room containing different extramaze cues. Each arm was 36.5 cm long and 7 cm wide, separated by a triangular center with 7-cm sides; all arms were enclosed by a wall of 12.5 cm height. Allocation of the start arm was counterbalanced within experimental groups. Time spent in the three arms was measured and used to obtain an index of percent novelty preference as follows: time spent in the novel arm/time spent in the three arms x 100. The maze was cleaned with 70% ethanol then water and wiped with paper towels between each trial.

Magnetic Resonance Spectroscopy (MRS)

MRS was realized as described previously with slight modifications [2]. Ten *Fmr1* KO and ten WT mice, which were not used in the behavioral tests, were included in the study. Mice were placed on a home-built custom to immobilize their head. They were anesthetized during magnetic resonance experiment with 1.5% isoflurane and a mixture O_2/N_2O (1:1) with an output of 0.7L / min. Respiration motion was controlled during all the experiment using an air pillow. Mice body temperature was maintained constant with a warm water circulation. MR spectroscopy was performed on a 7T horizontal magnet dedicated to small animal imaging

(70/16 USR Bruker Biospec, Wissembourg, Fr) and equipped with a 300mT/m gradient set. A Bruker 28 mm inner diameter bircage coil was used for both ^{1}H transmission and reception. First of all scout images were performed to localize the voxel of interest [10] using a RARE sequence with the following parameters: TR/TE = 5s / 56ms, rare factor = 8, FOV size = 2*2 cm, matrix size = 256*256, slice thickness = 1mm to display (78*78) µm in plane resolution for 5 min duration. Static B_0 homogeneity was adjusted with first and second order shims in a (2.5*2.5*2.5) mm voxel centred in the hippocampus (bregma: -2.10 mm) with Bruker Fastmap procedure[11]. The line width achieved for tissue water was less than 10 Hz. A PRESS sequence (Point Resolved Spectroscopy) was used to record localized ^{1}H spectra in a cubic (2*2*2) mm voxel placed in fast map voxel with the following parameters (TR = 4s, TE = 16ms, 256 scan : 17min, 2048 points, bandwidth = 4000Hz) with water suppression using VAPOR (VAriable Pulse power and Optimized Relaxation delays) module and outer volume suppression[12]. Eddy current compensation and static magnetic field drift correction were applied during spectra acquisition. ^{1}H spectra were collected for WT and *Fmr1* KO pre and post treatment. Spectra were analyzed with JMRUI 3.0 software (http://www.mrui.uab.es/mrui/) working in time domain (baseline correction, phasing, zero filling.). AMARES module [13] was used to quantify brain metabolites: glutamate, myo-inositol, N-Acetyl-Aspartate, taurine and lactate. Metabolites concentrations are represented in arbitrary unit (AU), after normalization by Creatine/Phosphocreatine, in box plots (the minimum, 1^{st} quartile, mean, median, 2^{nd} quartile and maximum).

Proteins extractions and Expression (Western blot)

Proteins extraction from murine neurons culture or human lymphoblastoïd cells were homogenized in ice-cold RIPA buffer (150mM NaCl, 50 mM TrisHCl, 5 mM EDTA, 0.1% SDS) supplemented with protease inhibitors (1% Protease inhibitor mixture, Fisher Scientific,

Illkirch, France). Protein concentration was determined by Pierce™ BCA pierce kit (ThermoScientific, France).

Protein extracts from murine neurons culture or human lymphoblastoïd cells (15µg) samples were separated in 12% SDS-PAGE, transferred to nitrocellulose membrane, incubated with 1:1000 diluted rabbit polyclonal anti-Kcnma1/anti-KCNMA1 antibody (Alomone Labs, France) or anti-FMRP antibody (ABCam, Paris, France) recognized by peroxydase-conjugated goat anti-rabbit antibodies. Load of equal amounts of protein was checked by 1:40000 diluted mouse anti-GAPDH antibody (ABCam, Paris, France). Quantification of immunoreactive bands was done using ImageJ (http://rsbweb.nih.gov/ij/). Blots were performed three times.

References

1. Ethell IM, Irie F, Kalo MS, Couchman JR, Pasquale EB, Yamaguchi Y: **EphB/syndecan-2 signaling in dendritic spine morphogenesis.** *Neuron* 2001, **31:**1001-1013.
2. Hering S: **Small-volume and rapid extracellular solution exchange around Xenopus oocytes during voltage-clamp recordings.** *Pflugers Arch* 1998, **436:**303-307.
3. Gribkoff VK, Starrett JE, Jr., Dworetzky SI: **Maxi-K potassium channels: form, function, and modulation of a class of endogenous regulators of intracellular calcium.** *Neuroscientist* 2001, **7:**166-177.
4. Jensen BS: **BMS-204352: A Potassium Channel Opener Developed for the Treatment of Stroke.** *CNS Drug Reviews* 2002, **8:**353-360.

5. Nadler JJ, Moy SS, Dold G, Trang D, Simmons N, Perez A, Young NB, Barbaro RP, Piven J, Magnuson TR, Crawley JN: **Automated apparatus for quantitation of social approach behaviors in mice.** *Genes Brain Behav* 2004, **3:**303-314.

6. 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:**e17073.

7. Dellu F, Contarino A, Simon H, Koob G, Gold L: **Genetic differences in response to novelty and spatial memory using a two-trial recognition task in mice.** *Neurobiology of Learning and Memory* 2000, **73:**31-48.

8. Dellu F, Fauchey V, Moal ML, Simon H: **Extension of a new two-trial memory task in the rat: influence of environmental context on recognition processes.** *Neurobiology of learning and memory* 1997, **67:**112-120.

9. Dellu F, Mayo W, Cherkaoui J, Le Moal M, Simon H: **A two-trial memory task with automated recording: study in young and aged rats.** *Brain research* 1992, **588:**132-139.

10. Voineagu I, Wang X, Johnston P, Lowe JK, Tian Y, Horvath S, Mill J, Cantor RM, Blencowe BJ, Geschwind DH: **Transcriptomic analysis of autistic brain reveals convergent molecular pathology.** *Nature* 2011, **474:**380-384.

11. Gruetter R, Fusch C, Martin E, Boesch C: **Determination of saturation factors in 31P NMR spectra of the developing human brain.** *Magn Reson Med* 1993, **29:**7-11.

12. Tkac I, Starcuk Z, Choi IY, Gruetter R: **In vivo 1H NMR spectroscopy of rat brain at 1 ms echo time.** *Magn Reson Med* 1999, **41:**649-656.

13. Vanhamme L, van den Boogaart A, Van Huffel S: **Improved method for accurate and efficient quantification of MRS data with use of prior knowledge.** *J Magn Reson* 1997, **129:**35-43.

Supplementary Results

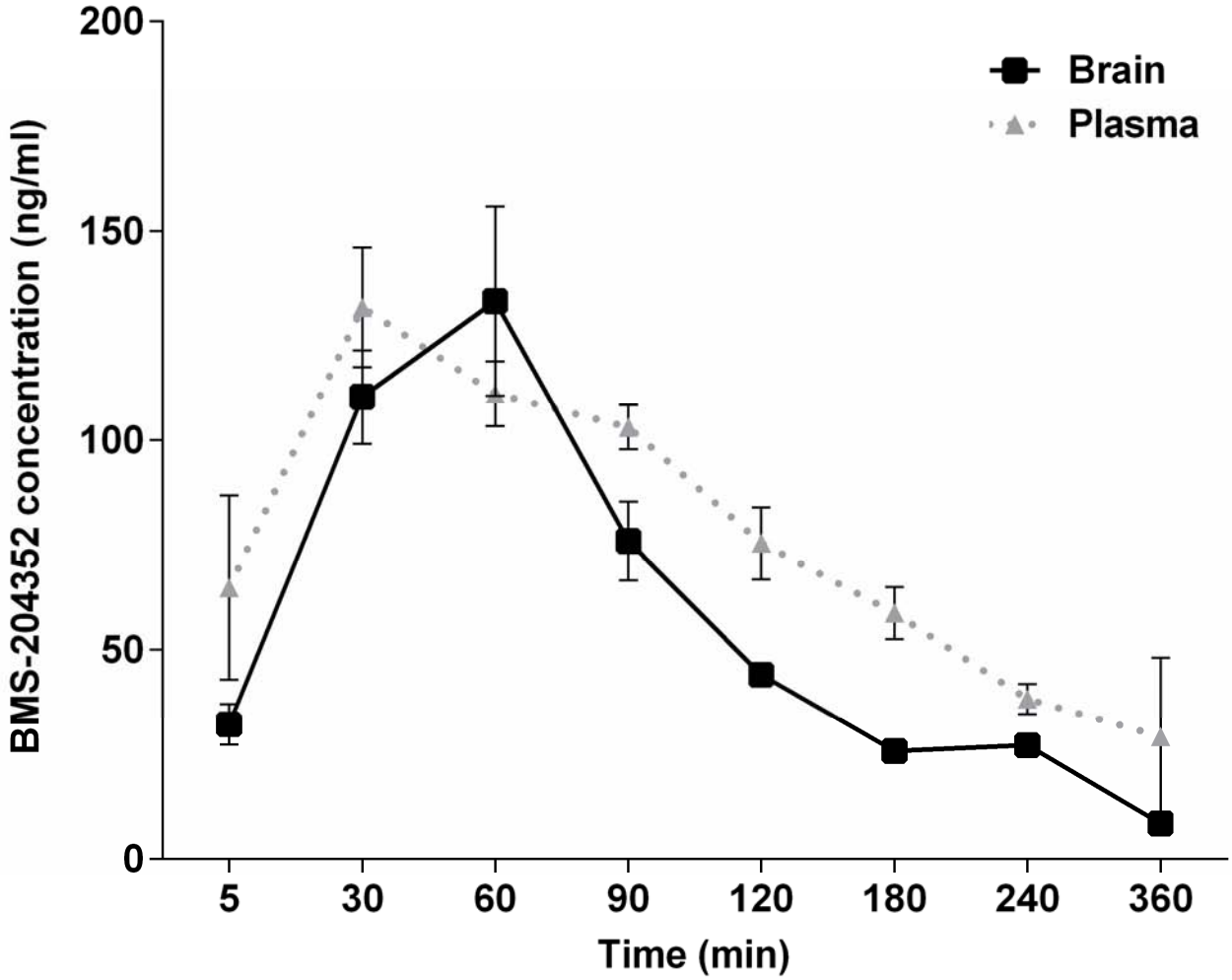

Figure S1. BMS-204352 2mg/kg pharmacokinetic results. Following intraperitoneal administration at 2mg/kg, plasma concentration *versus* time curve increased to an observed Cmax 139 ± 27 ng.mL^{-1} with a Tmax 30 min. A slightly delayed concentration time-course were observed in the brain (Tmax = 42 ± 16 min) with a Cmax 142 ± 43 ng.mL^{-1} but with an earlier decrease than in plasma. Tmax and Cmax were not statistically different between plasma and brain ($p < 0.05$). Mean BMS-204352 concentrations ± s.e.m. in plasma (▲, dashed line) and in brain (■, solid line) *versus* time in mice after an intraperitoneal bolus of 2mg/kg of BMS-204352.

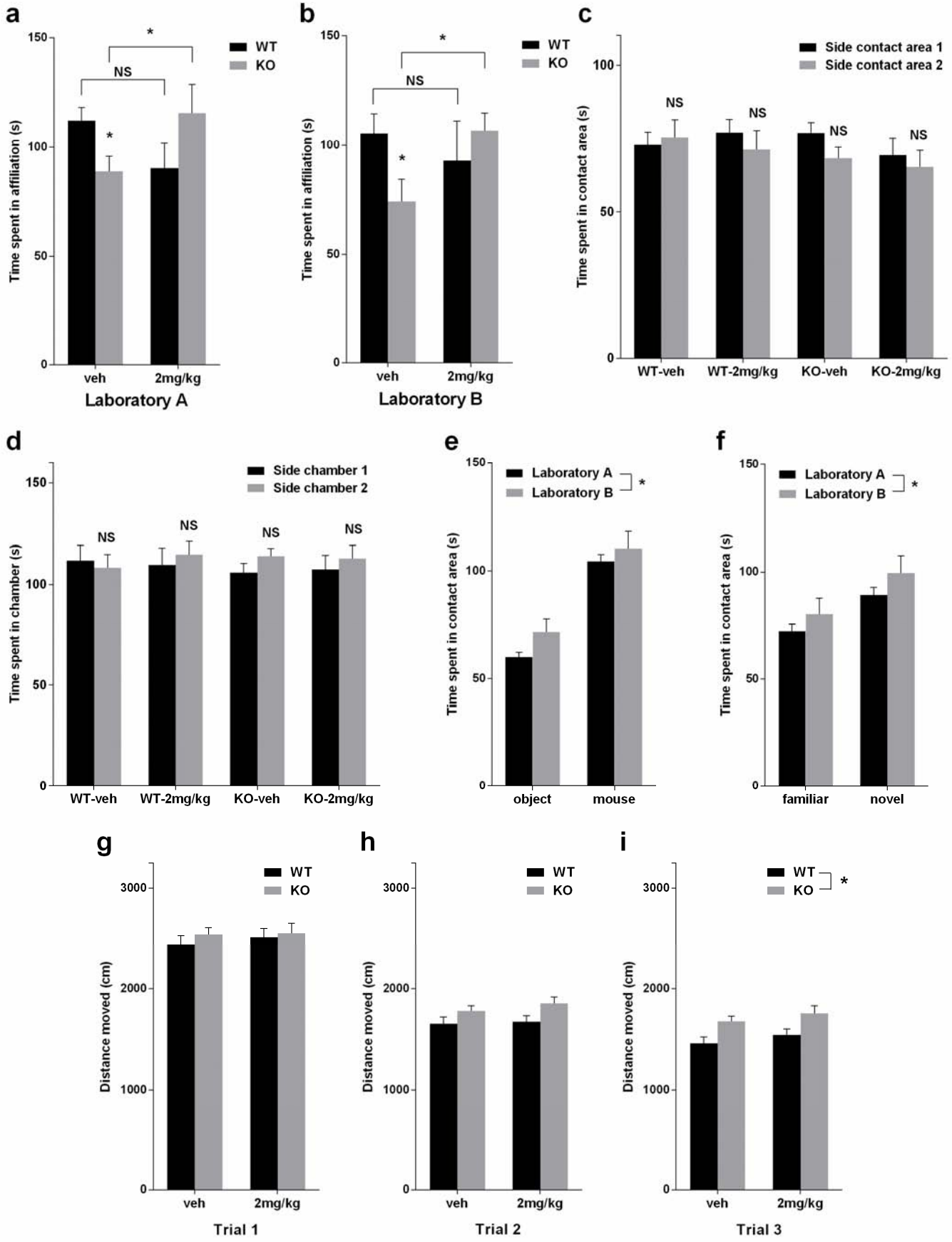
a
b
c
d
e
f
g
h
i
WT
KO
Time spent in affiliation (s)
NS
*
veh
2mg/kg
Laboratory A
Laboratory B
Side contact area 1
Side contact area 2
Time spent in contact area (s)
WT-veh
WT-2mg/kg
KO-veh
KO-2mg/kg
Side chamber 1
Side chamber 2
Time spent in chamber (s)
Laboratory A
Laboratory B
object
mouse
familiar
novel
Distance moved (cm)
Trial 1
Trial 2
Trial 3

Figure S2. BMS-204352 effects on Social behaviors. Mice (3-5 months) were administered with BMS-204352 2mg/kg or vehicle (veh) and subjected to social behavioral tests 30 min after the injection. (**a-b**) Time spent in affiliative behaviors (sum of nose, body, and anogenital sniffing and allogrooming) during the direct social interaction test from Laboratory A and Laboratory B. Vehicle-treated *Fmr1* KO mice spent less time in affiliative behavior compared to WT, but BMS-204352 injection restored a normal social investigation. (*n*: WT-veh = 20; WT-2mg/kg = 10; KO-veh = 17; KO-2mg/kg = 10 from Laboratory A; WT-veh = 7; WT-2mg/kg = 6; KO-veh = 8; KO-2mg/kg = 7 from Laboratory B). (**c**) Preference for a side in trial 1 (habituation) during the three-chamber test was measured by the time spent in the compartments and (**d**) the contact areas. (**e-f**) MANOVA indicated that all experimental groups did not show a preference for any chamber in both laboratories. (**g-h-i**) Distance moved along trials. Main effect of laboratories observed during trial 2 and trial 3 without interaction between genotype, treatments and contact areas. NS, not significant; *$p < 0.05$ (*n*: WT-veh = 28; WT-2mg/kg = 13; KO-veh = 34; KO-2mg/kg = 17 from Laboratory A; WT-veh = 6; WT-2mg/kg = 6; KO-veh = 8; KO-2mg/kg = 8 from Laboratory B). Data represent mean ± s.e.m.

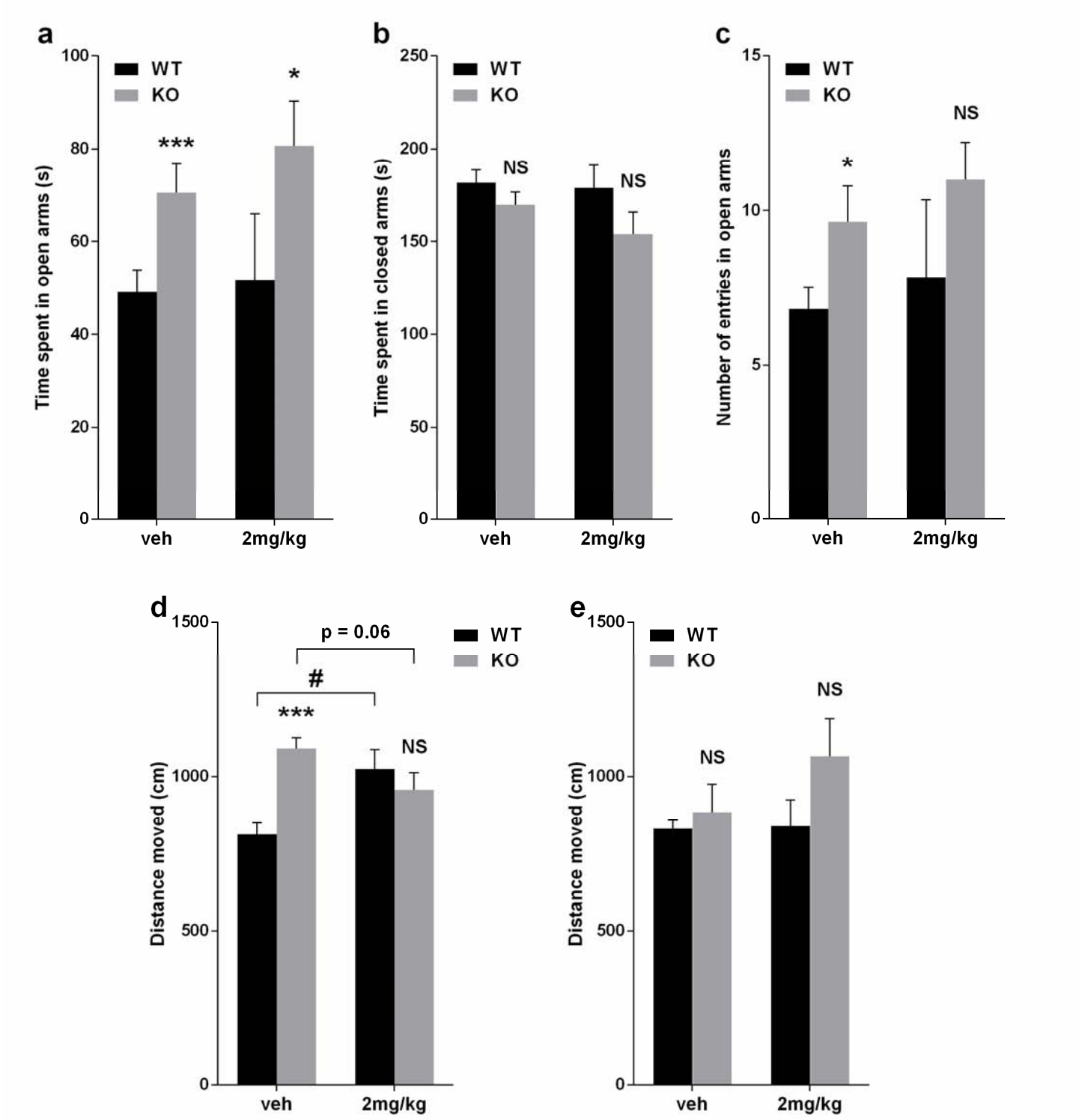

Figure S3. BMS-204352 effects on Anxiety in the elevated plus maze and locomotion during the spatial memory Y maze test. Time spent in (**a**) open arms and (**b**) closed arms was counted when mice were subjected to the elevated plus maze, (**c**) number of entries in open arms and (**d**) the distance moved. Significance was determined using two-way ANOVA with *post-hoc* PLSD test. NS, not significant; *$p < 0.05$, ***$p < 0.001$ and $^{\#}p < 0.05$ (*n*: WT-veh = 32; WT-2mg/kg = 7; KO-veh = 32; KO-2mg/kg = 10). (**e**) Distance moved during the 5min-Y maze test. Significance was determined using two-way ANOVA with *post-hoc* PLSD test. NS, not significant (*n*: WT-veh = 7; WT-2mg/kg = 6; KO-veh = 7; KO-2mg/kg = 7). Data represent mean ± s.e.m.

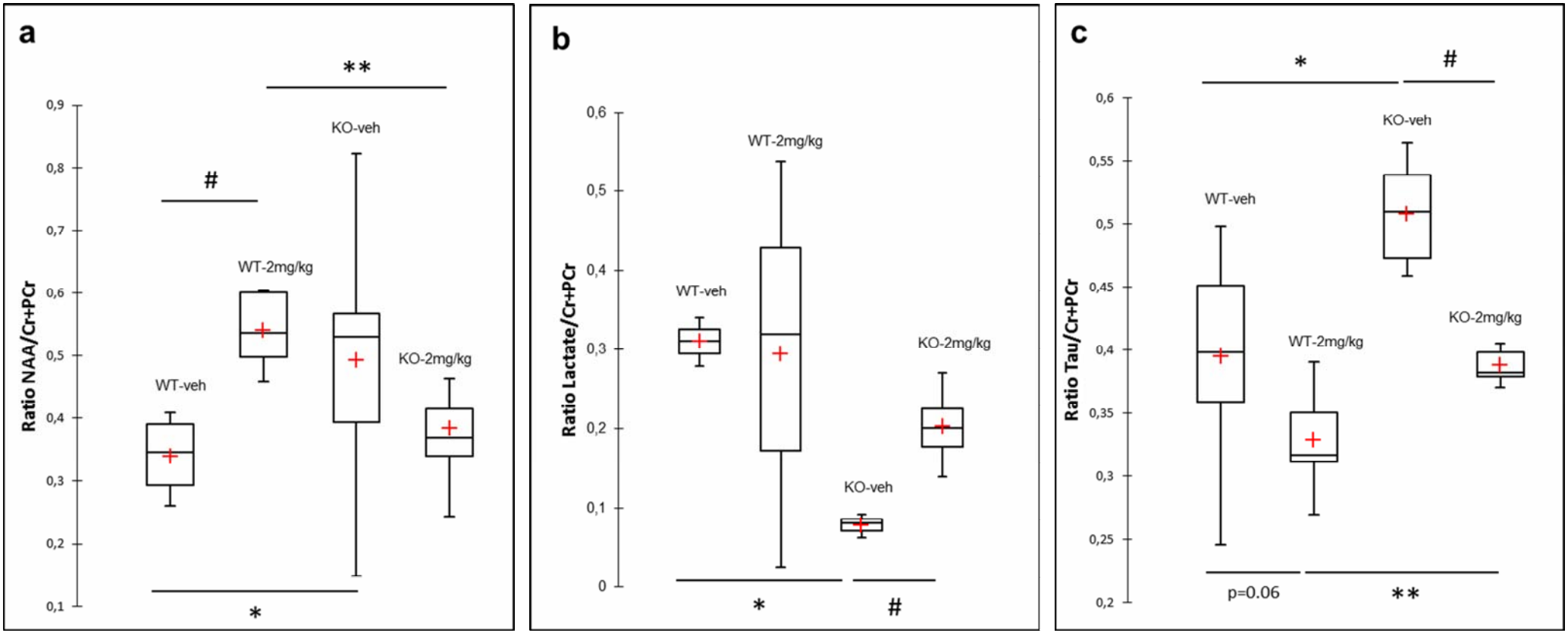

Figure S4. BMS-204352 effects on N-Acetyl-Aspartate, lactate and taurine brain concentration. Mice (3-5 months) were administered with BMS-204352 at 2 mg/kg or vehicle (veh) and subjected to MRS test after 30 min from the injection. Box plots (shown as the minimum, 1st quartile, mean, 2nd quartile and maximum values) represent metabolite concentration in arbitrary unit (AU) (**a**) N-Acetyl Aspartate, (**b**) lactate and (**c**) taurine, in vehicle-treated WT and KO as in BMS-204352 treated WT and KO. Significance between WT and KO was determined by pairs using Mann-Whitney test and impact of treatment was determined by Wilcoxon test. NS, not significant; *$p < 0.05$, **$p < 0.01$, compared to WT; $^{\#}p < 0.05$ compared to treatment.

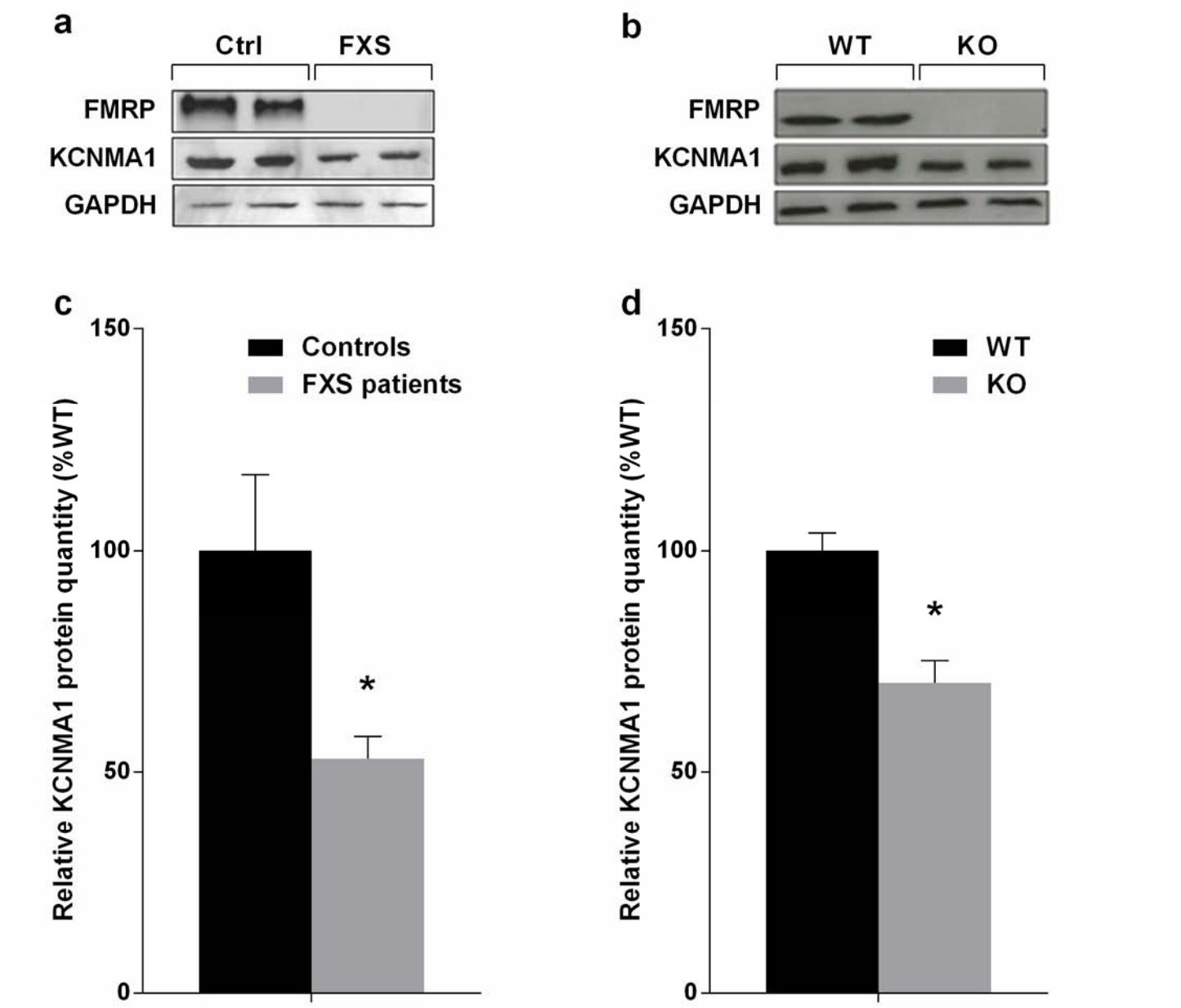

Figure S5. KCNMA1 protein decreased in human FXS patients and in *Fmr1* KO mice. Protein quantity was measured by western-blot (normalized by GAPDH housekeeping protein) in (**a**) human lymphoblastoid cells (Controls and FXS patients) as in (**b**) primary neurons culture of mice (WT and *Fmr1* KO). (**C-D**) In human FXS patients as in primary neurons culture of *Fmr1* KO mice, KCNMA1 is significantly decreased by ~ 40%. Significance was determined using one-way ANOVA with post-hoc PLSD test. **P*<.05; n = 10 mice per groups and n = 5 human individuals per groups. Error bars represent s.e.m.

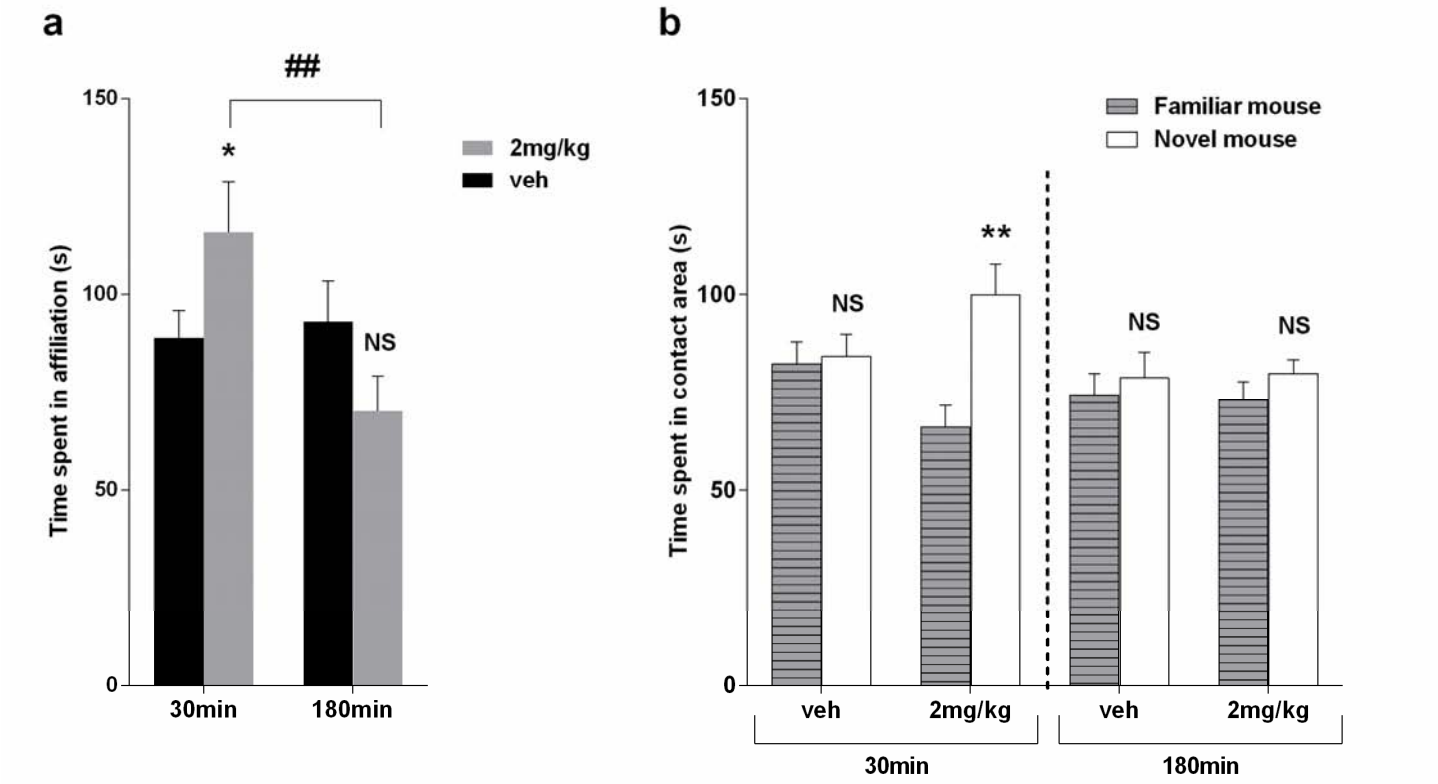

Figure S6. Long time BMS-204352 effects on Social behaviors. ***Fmr1*** **KO** mice (3-5 months) were administered with BMS-204352 2mg/kg or vehicle (veh) and subjected to social behavioral tests 30 min or 180 min after the injection. (**a**) Time spent in affiliative behaviors (sum of nose, body, and anogenital sniffing and allogrooming) during the direct social interaction test. BMS-204352 at 30min after injection restored a normal social investigation but not after 180 min. NS, not significant; *$p < 0.05$, $^{\#\#}p < 0.01$. (**b**) Preference for a novel versus a familiar mouse in trial 3 (social novelty) during the three-chamber test, was measured by the time spent in the contact area when a stranger and a familiar mouse were accessible. Vehicle-treated *Fmr1* KO mice didn't present a preference for the novel mouse. This preference was recue by BMS (2mg/kg) at 30 min delay after injection but not at 180 min. NS, not significant; **$p < 0.01$ (familiar versus novel mouse). (*n*: 30min : KO-veh = 34; KO-2mg/kg = 17 ; 180min : KO-veh = 10; KO-2mg/kg = 10). Data represent mean ± s.e.m.