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Complete List of Authors:	Hurley, Michael; University of Southampton, Biological Sciences; UCL Queen Square Institute of Neurology, Clinical and Movement Neurosciences Deacon, Robert; Fraunhofer Research Institute, Biomedicine Division Chan, Edith; Wolfson Institute for Biomedical Science, Biological and Medicinal Chemistry Baker, David; Institute of Cell and Molecular Science, Queen Mary University of London, Neuroscience Centre Selwood, david; Wolfson Institute for Biomedical Science, 5Biological and Medicinal Chemistry Cogram, Patricia; Fraunhofer Research Institute, Biomedicine Division; Universidad de Chile, Ecological Sciences
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Reversal of behavioural phenotype by the cannabinoid-like compound VSN16R in fragile X syndrome mice

Michael J Hurley[1,2], Robert MJ Deacon[3], AW Edith Chan[4],
David Baker[5], David L Selwood[4], Patricia Cogram[3,6]

Abstract

Fragile X Syndrome is the most common inherited intellectual disability and mono-genetic cause of autism spectrum disorder. It is a neurodevelopmental condition occurring due to a CGG trinucleotide expansion in the *FMR1* gene. Polymorphisms and variants in large-conductance calcium-activated potassium channels are increasingly linked to intellectual disability and loss of FMR protein caused reduced large-conductance calcium-activated potassium channel activity leading to abnormalities in synapse function. Using the cannabinoid-like large-conductance calcium-activated potassium channel activator VSN16R we rescued behavioural deficits such as repetitive behaviour, hippocampal dependent tests of daily living, hyperactivity and memory in a mouse model of fragile X syndrome. VSN16R has been shown to be safe in a phase 1 study in healthy volunteers and in a phase 2 study in people with Multiple Sclerosis with high oral bioavailability and no serious adverse effects reported. VSN16R could therefore be directly utilised in a fragile X syndrome clinical study. Moreover, VSN16R showed no evidence of tolerance, which strongly suggests that chronic VSN16R may have great therapeutic value for fragile X syndrome and autism spectrum disorder. This study provides new insight into the pathophysiology of fragile X syndrome and identifies a new pathway for drug intervention for this debilitating disorder.

Author affiliations:

1 Neuroimmunology, Biological Sciences, Faculty of Environmental and Life Sciences, University of Southampton, Southampton SO16 6YD, UK

2 Department of Clinical and Movement Neurosciences, UCL Queen Square Institute of Neurology, London NW3 2PF, United Kingdom

3 Biomedicine Division, Fraunhofer Research Institute, Center for System Biotechnology, Santiago, Chile

4 Drug Discovery, UCL Wolfson Institute for Biomedical Research, University College London, London WC1E 6BT, UK

5 Centre for Neuroscience and Trauma, Blizard Institute, Queen Mary University of London, London E1 2AT, UK

6 Department of Ecological Sciences, Institute of Ecology and Biodiversity, Faculty of Sciences, Universidad de Chile, Santiago, Chile

Correspondence to: Patricia Cogram or David Selwood.

P. Cogram, Department of Ecological Sciences, Institute of Ecology and Biodiversity, Faculty of Sciences, Universidad de Chile, Santiago, Chile

D. Selwood, Drug Discovery, UCL Wolfson Institute for Biomedical Research, University College London, London WC1E 6BT, UK

E-mail: patricia.cogram@gmail.com, d.selwood@ucl.ac.uk

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Introduction

Fragile X Syndrome (FXS) is an inherited neurodevelopmental condition characterised by intellectual disability, hyperactivity/hyperkinesis, attention deficits, social difficulties, anxiety, depression, irritability, mania, obsessive-compulsive behaviour, aggression, self-injury and autistic-like behaviours.[1] It is caused by a CGG trinucleotide expansion in the fragile X mental retardation 1 (*FMR1*) gene on the X chromosome at locus Xq27.3 that leads to lowered expression of fragile X mental retardation (FMRP) protein.[2-5] FMRP is essential in neurodevelopment and for normal synaptic function in adult brain.[6] There is currently no specific cure or effective disease-modifying treatment for FXS.

FMRP interacts with large-conductance voltage and calcium -activated potassium (BK) channels to control their expression and function.[7] BK channels consist of alternatively spliced isoforms of four pore-forming, voltage- and calcium-sensing alpha-subunits (KCNMA1) encoded by a single gene usually in association with accessory transmembrane beta- (KCNMB1-4) and gamma- subunits, which change the kinetic, pharmacological and membrane-trafficking properties of the BK channel complex.[8,9] BK channels are widely expressed on neuronal and smooth muscle cells and undergo complex post-translational modification.[10]

Laumonnier and colleagues[11] first reported reduced BK channel function in autism and intellectual disability in a subject with a haploinsufficiency in KCNMA1-alpha. In FXS, the first indication of a reduction of BK channel activity was reported by Tang and colleagues[12] who showed that protein levels of *Kcnma1*-alpha in *Fmr1* KO synaptic fractions were reduced by about 50%, resembling the haploinsufficiency reported by Laumonnier and colleagues[11]. Hébert and colleagues[13] showed that the BK channel opener BMS-204352 rescued the FXS phenotype in *Fmr1* KO mice. The range of BK channel polymorphisms affecting intellectual disability and FXS have recently been expanded and reported by many different groups.[7]

VSN16R is an orally active cannabinoid-like compound that activates BK channels in brain that is well-tolerated, has high oral bioavailability and no adverse neurobehavioural effects in rodents or humans.[14] VSN16R was shown to be safe in a phase 1 study of healthy individuals[15] and completed a phase II trial (NCT02542787) for spasticity.[16] VSN16R showed no evidence of tolerance and may have therapeutic value for FXS and autism spectrum disorder, so this study sought to evaluate the effect of chronic treatment with VSN16R on the

FXS-like behavioural phenotype of *Fmr1* KO2 mice that exhibit many of symptoms found in FXS patients.[17]

VSN16R rescued behavioural deficits caused by FMRP loss such as repetitive behaviour, hippocampal dependent tests of daily living, hyperactivity, memory impairment, stereotypy and aggression. In addition, we collated the existing data on polymorphisms and intellectual disability in the BK channel and mapped these onto the BK channel cryo-EM crystal structure. Published gene expression data for the BK channel show that the isoforms of the BK channel activated by VSN16R are found in areas of the brain that are associated with FXS. This supports the role of BK channels in the cause of FXS symptoms and modulation of BK channel function as a therapy for treatment of FXS.

Material and Methods

Animals

Fmr1 KO2 mice supplied by the FRAXA Research Foundation were used in this study. Experiments were conducted in line with the requirements of the UK Animals (Scientific Procedures) Act, 1986 and were approved by the ethics committee of the Institute of Ecology and Biodiversity, Faculty of Sciences, University of Chile.

Animal housing

Mice were housed in groups of 5 in plastic cages (35 x 30 x 12 cm) in a controlled environment (21 ± 2°C, relative humidity (55 ± 5%), 12-h light–dark cycle (lights on 7 a.m.–7 p.m.) and air exchange 16 times per h) with free access to commercial food pellets and water.

Treatment groups

Before chronic dosing with VSN16R (R,Z)-3-(6-(dimethyl-amino)-6-oxohex-1-en-1-yl)-N-(1-hydroxypropan-2-yl)-benzamide (Canbex therapeutics Ltd.), 2 mg/kg iv, qd, in 0.9% NaCl, 4 weeks), mice were challenged with a single intravenous dose of VSN16R and monitored for adverse behavioural or physiological effects. VSN16R was well tolerated by *Fmr1* KO2 and WT mice consistent with other toxicological studies (Baker et al. 2017).[15] Following chronic dosing, 14-week-old *Fmr1* KO2 and WT mice were tested within 30 min of VSN16R

administration in the following groups: WT + Vehicle; WT + VSN16R; *Fmr1* KO2 + Vehicle and *Fmr1* KO2 + VSN16R (n = 10).

Behavioural tests

Behavioural testing was conducted between 8 am – 4 pm as previously described.[18] Hence any effects of the BK channel on circadian pacemaker control would be the same for all animals used in the study. Mice were randomly assigned to treatment groups. The behavioural experimenter was blind to genotype, drug treatment and subsequent data analysis. Mice were tested in one behavioural task on each experimental day and each behavioural test was separated by 3 days.

Open field

The open field was used to determine hyperactivity and habituation to a novel environment - one of the most elementary forms of learning - in which decreased exploration as a function of repeated exposure to the same environment (an enclosed arena 50 x 30 cm divided into 10 cm squares) was taken as an index of memory. Testing consisted of an initial exposure (T1), then at 10-min (T2) to test short-term memory and after 24hr (T3) to assess long-term memory.

Contextual fear conditioning

Fear conditioning to a context tests associative learning. Mice were given a 120 s habituation period in the apparatus before the first of two identical trials (210 s apart) to allow exploration of the chamber. An 80 dB auditory cue was then presented (15-30 s) with a mild foot shock (0.6 mA, 1 s) administered during the last 2 sec of the tone presentation, that co-terminated with the tone. Memory was tested 24 h after training for 5 min.

Marble burying

Transparent plastic cages were filled (10-cm deep) with sawdust, on top of which 10 glass marbles were placed in two rows. Each animal was left undisturbed in the cage for 30 min, after which the number of marbles buried to at least 2/3 of their depth was recorded.

Self-grooming

Mice were placed individually in a cage (46 × 23.5 × 20 cm) illuminated at 40-lux. After a 5-min habituation period the time spent grooming was recorded for 3 min.

Aggression

An experimental 'test' and WT 'control' mouse were placed in the testing cage simultaneously. The latency to attack was recorded.

BK channel and FMRP modelling and polymorphism mapping

Numerous BK channel polymorphisms are linked with intellectual disability (Supplementary Table 1). A Molecular Operating Environment modelling package (Chemical Computing Group) was used to analyse the BK channel cryo–EM structure[19] and visualise the location of polymorphisms.

BK channel expression in rodent and human brain

Expression data was obtained from public repositories to compare gene expression of FRMP and the BK channel isotypes in human and rodent brain (Supplementary Figures 1 and 2).

Data analysis

Data were analysed using SPSS™ statistics software v.27 (IBM) and visualized with Prism™ v.9 (GraphPad).

Data Availability

All data generated or analysed and used in this study are available upon reasonable request.

Results

Behavioural tests

Open field

The open field trial 1 (T1) was performed to characterize hyperactivity in *Fmr1* KO2 and WT littermates habituated to daily handling under novelty and familiar conditions. *Fmr1* KO2 mice injected with vehicle displayed a significant increase in total distance traveled (a parameter for hyperactivity) compared to the WT vehicle-control group. In contrast, the *Fmr1* KO2 mice injected with VSN16R had a significantly reduced total distance traveled compared to the *Fmr1* KO2 mice injected with vehicle and displayed similar activity to the WT control group treated with vehicle or VSN16R that indicated chronic VSN16R treatment decreased spontaneous hyperactivity occurring in *Fmr1* KO2 mice (Figure 1A).

The open field trial 2 (T2) test assessed short-term memory. All groups other than the vehicle treated *Fmr1* KO2 had a lower activity, indicating habituation (i.e. memory of the environment), than was observed in the T1 test. Whereas vehicle treated *Fmr1* KO2 mice exhibited significantly increased locomotor activity compared to WT vehicle treated mice indicating that they did not remember having explored the open field before and therefore had a short-term memory problem. The increased locomotor activity was significantly reduced (i.e. short-term memory deficit was corrected) in the *Fmr1* KO2 mice injected with VSN16R compared to the vehicle treated *Fmr1* KO2 mice to a level similar to that occurring in the wild-type animals (Figure 1B).

The open field trial 3 (T3) test assessed long-term memory of the open-field environment. The exploratory behavior was again lower in all groups apart from the vehicle treated group *Fmr1* KO2 group that showed significantly less habituation (i.e. more activity) indicative of reduced long-term memory of the environment. (Figure 1C).

Contextual fear conditioning

Freezing in response to an aversive stimulus (a measure of associative learning) was significantly less in *Fmr1* KO2 mice treated with vehicle compared to wild-type animals treated with vehicle or VSN16R. Chronic treatment with VSN16R significantly reduced the fear conditioning response in *Fmr1* KO2 mice although it was still significantly less than the response seen in wild-type animals (Figure 1D).

Marble burying

Marble burying (analogous to activities of daily living in humans) was significantly reduced in *Fmr1* KO2 compared to WT animals and chronic VSN16R treatment significantly restored this behaviour (Figure 1E).

Stereotypy

Self-grooming behaviour was significantly higher in *Fmr1* KO2 mice compared to wild-type animals and was reduced in *Fmr1* KO2 mice treated with VSN16R to a similar level to that found in wild-type animals (Figure 1F).

Aggression

The time before attack was significantly shorter in *Fmr1* KO2 mice compared to wild-type animals which indicated increased aggression and was increased in *Fmr1* KO2 mice treated with VSN16R to a similar level to that found in wild-type animals (Figure 1G).

BK channel and FMRP modelling and polymorphism mapping

The location of polymorphisms in the BK channel[11,19] and FMRP[20] that give rise to a clinical phenotype are shown in Figure 2.

Discussion

The behavioural findings described in this study provide direct evidence that chronic treatment with VSN16R, a selective activator of BK channels, can rectify the hyperactivity, short-term and long-term memory deficits and reduce stereotypy and aggression that occur in the *Fmr1* KO2 mouse model of FXS. This effect is likely to be due to activation of BK receptors on neurons in brain regions involved in these behaviours (as opposed to smooth muscle) and the *in silico* analysis of published BK receptor expression studies supported this view (Supplementary Figures 2 and 3). Consequently, the drug may also be of use in patients with FXS since there is much overlap with expression of the BK receptor between mice and humans.

A range of tests were used to explore the effect of VSN16R on FXS-like behaviours in the mouse model of the disorder. Marble burying behaviour in mice is analogous to activities of daily living in humans. These impairments are frequently more of a problem to the patient than the loss of more complex cognitive abilities.[21] VSN16R treatment restored marble burying to control levels in *Fmr1* KO2 mice. There is debate on whether this test measures anxiety or obsessive-compulsive disorder-like behaviours and hence which symptoms of FXS patients it relates to. However, it does show sensitivity to anxiolytic compounds and drugs that affect the serotonergic system and since *Fmr1* KO2 mice do less marble burying, indicating less repetitive (obsessive-compulsive like) actions in this test, it is likely that marble burying behaviour is more a measure of anxiety than obsessive-compulsive behaviour.[22] However, Bhattacharya and colleagues[23] reported increased marble burying in *Fmr1* KO mice, which suggested an opposite action. Marble burying are species-typical behaviours that have been shown to be sensitive to animal species, strain, hippocampal lesions, thus, difference between *Fmr1* KO and *Fmr1* KO2 models or subtle differences in the methods employed (e.g. marble burying depth) could affect this test outcome.

Hyperactivity is a confounding factor in the *Fmr1* KO mouse models, nevertheless, fear conditioning has an amygdala and hippocampal element. VSN16R reduces *Fmr1* KO2 locomotor activity but also improves hippocampal dependent activities of daily living, indicating that it has a role in improving hippocampal functions, like memory, beyond simple improvement of locomotor tasks. *Fmr1* KO2 mice show a significant deficit in contextual fear conditioning memory to an aversive stimulus compared with wild-type littermate mice and treatment with VSN16R before acquisition improved long-term memory retention 24 hr after training. VSN16R could have improved memory retention by enhancing memory acquisition, memory consolidation or both processes. However, this effect could have been due to reductions in hyperactivity as were seen in the open field tests although, it is important to take into consideration that VSN16R also improves hippocampal dependent activities of daily living, indicating that it has a role in improving hippocampal functions, like memory, beyond simple improvement of locomotor tasks.

Fmr1 KO2 mice also showed increased self-grooming compared with WT littermates. One possible interpretation is that *Fmr1* KO2 animals are more prone to repetitive action patterns (i.e., stereotypy). VSN16R decreased stereotyped self-grooming behaviour in *Fmr1* KO2 mice suggesting that this task could be more sensitive to the drug. This finding seemingly contradicts the observation of reduced marble burying in *Fmr1* KO2 described above. However, previous hippocampal lesion studies showed that lesioned mice displayed reduced marble burying behaviour with little change in self grooming which suggests that these behaviours are independently mediated.[22]

A recent study by Wheeler and colleagues[24] reported most patients-with FXS surveyed were aggressive in the previous 12 months and self-injury and impulsive behaviour are also more prevalent in individuals with FXS.[25] Aggressive behaviour was significantly increased in *Fmr1* KO2 mice and was reversed by chronic VSN16R treatment.

Studies examining expression of BK channels in *FmR1* KO mice are limited. RNAseq has been performed on *Fmr1* KO mice and differential *Kcnmb4* neuronal expression was found in some neuronal populations such as cortical neurons[26,27] but not others such as in the CA1 region of the hippocampus.[28,29]

We collated the published KCNMA variants and polymorphisms known to influence intellectual disability and mapped them onto the KCNMA/KCNMB4 cryo-EM structure revealing that most variants were in accessible cytoplasmic regions and therefore likely to

adversely affect BK channel function (Supplementary Table 1, Figure 2). The sequence context of variants is informative from a structural perspective for design of novel drugs and understanding the functional significance of polymorphisms. For example, the A138V variant when viewed in the context of the INNGSSQADG sequence introduces a large (valine) amino acid change in an otherwise polar region. Dysregulated interaction with FMRP, as highlighted by the R138Q FMRP variant, may disrupt BK channel function and an effect on intellectual disability can result. Unfortunately, the details of the interaction between BK channel and FMRP are not known at the present time. The distribution of BK channels in brain was consistent with VSN16R inhibiting spasticity, which is associated with stress induced hyper-excitation of glutamatergic nerves.[15] This suggested the potential for control of autism spectral disorders, and other conditions, that are likewise associated with an imbalanced extra and intra-neuronal environment that favours excitation over inhibition and glutamate-mediated hyper-excitability and can be regulated by BK channels.[13,27,30,31]

Importantly, VSN16R showed no evidence of tolerance after chronic treatment, which has been a problem with other drug classes used as putative treatments for FXS in patients and models of the disorder.[32] This, combined with the effective reversal of the FXS behavioural phenotype in the *Fmr1* KO2 mice means VSN16R may be of benefit in treating FXS and autism spectrum disorder related behaviours and is therefore a strong candidate for future clinical trials.

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Competing interests

The authors declare no competing interests. Patent WO2016128771A1 was filed by Canbex Therapeutics Ltd. in relation to use of VSN16R to treat multiple sclerosis and FXS.

Supplementary material

Supplementary material is available at *Brain* online.

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Figure 1. Behavioural effects of VSN16R on *Fmr1* KO2 mice

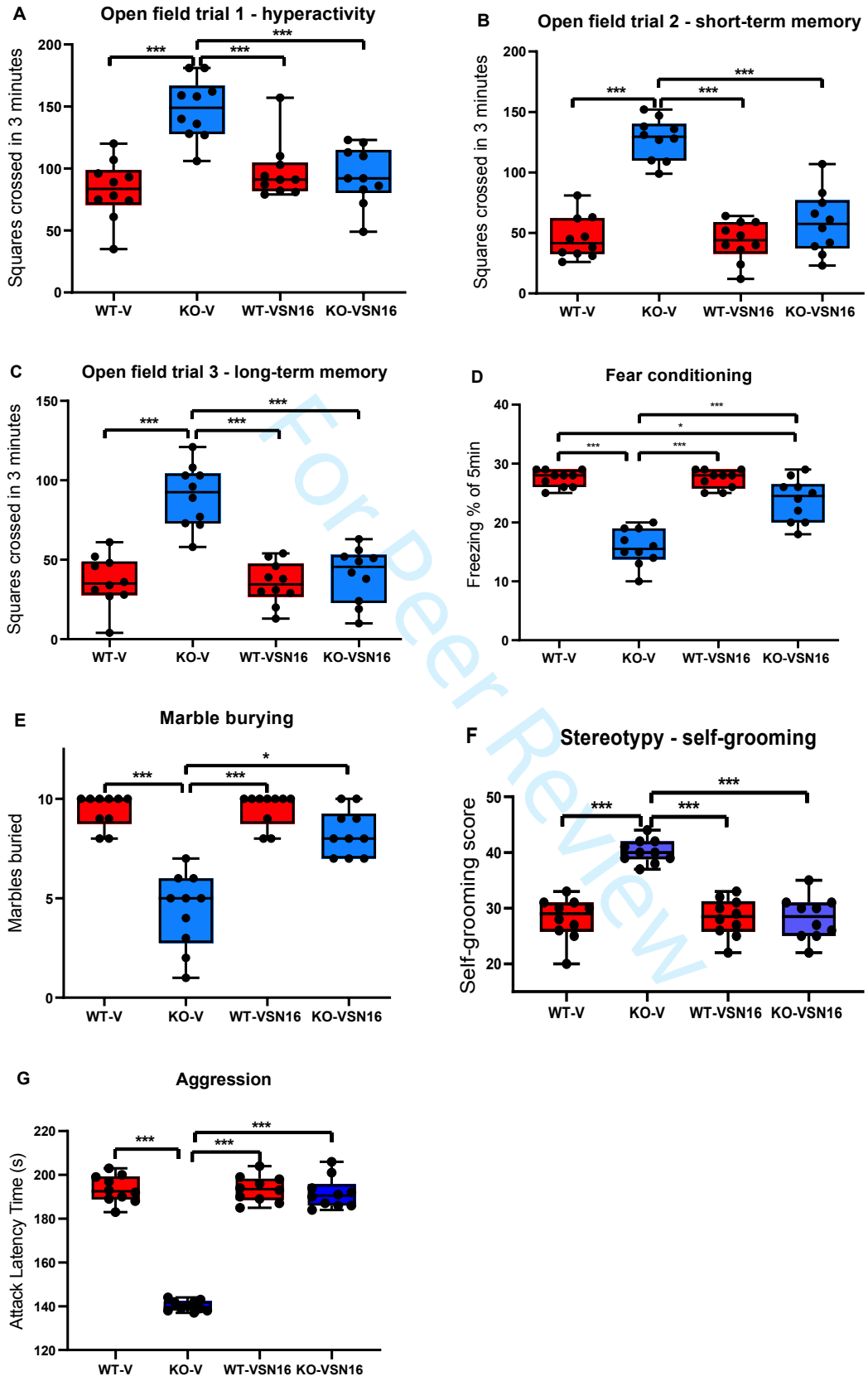

Figure 1. Wild-type (WT) or *Fmr1* KO2 (KO) adult mice were treated with vehicle (V) or VSN16R for 4 weeks and then subjected to a range of behavioural tests. VSN16R treatment caused significant improvement in (**A**) hyperactivity, (**B**) short term memory and (**C**) long-term memory in *Fmr1* KO2 mice when compared to WT littermates in the open field test. (**D**) VSN16R rescued impaired learning and memory of the *Fmr1* KO2 mice in the contextual fear conditioning test. (**E**) VSN16R reversed impaired marble burying activity, a behaviour analogous to activities of daily living in humans, of the *Fmr1* KO2 mice when compared to wild type littermates. (**F**) Self-grooming, a measure of stereotypy that occurs in *Fmr1* KO2 mice, was restored to levels found in wild-type animals by chronic VSN16R treatment and (**G**) the increased aggression that occurs in *Fmr1* KO2 mice was reversed by chronic VSN16R treatment. Data was visualized as boxplots with interquartile ranges, presenting all the data points. The distribution of behavioural data was assessed with a normality test. Data with a normal distribution (Shapiro-Wilk, $P > 0.05$ = normal distribution) were analysed by univariate general linear model one-way ANOVA (F) followed, where appropriate, by a Bonferroni corrected multiple pair-wise comparison. Marble burying failed normality testing and was analysed by the Kruskal-Wallis one-way ANOVA by ranks (H) with Bonferroni corrected multiple pair-wise comparisons. (n = 10 mice per group, * $P < 0.05$; *** $P < 0.001$).

Figure 2 Cryo-EM structure of KCNMA1/KCNMB4 complex (modelled from Tao & MacKinnon, 2019)

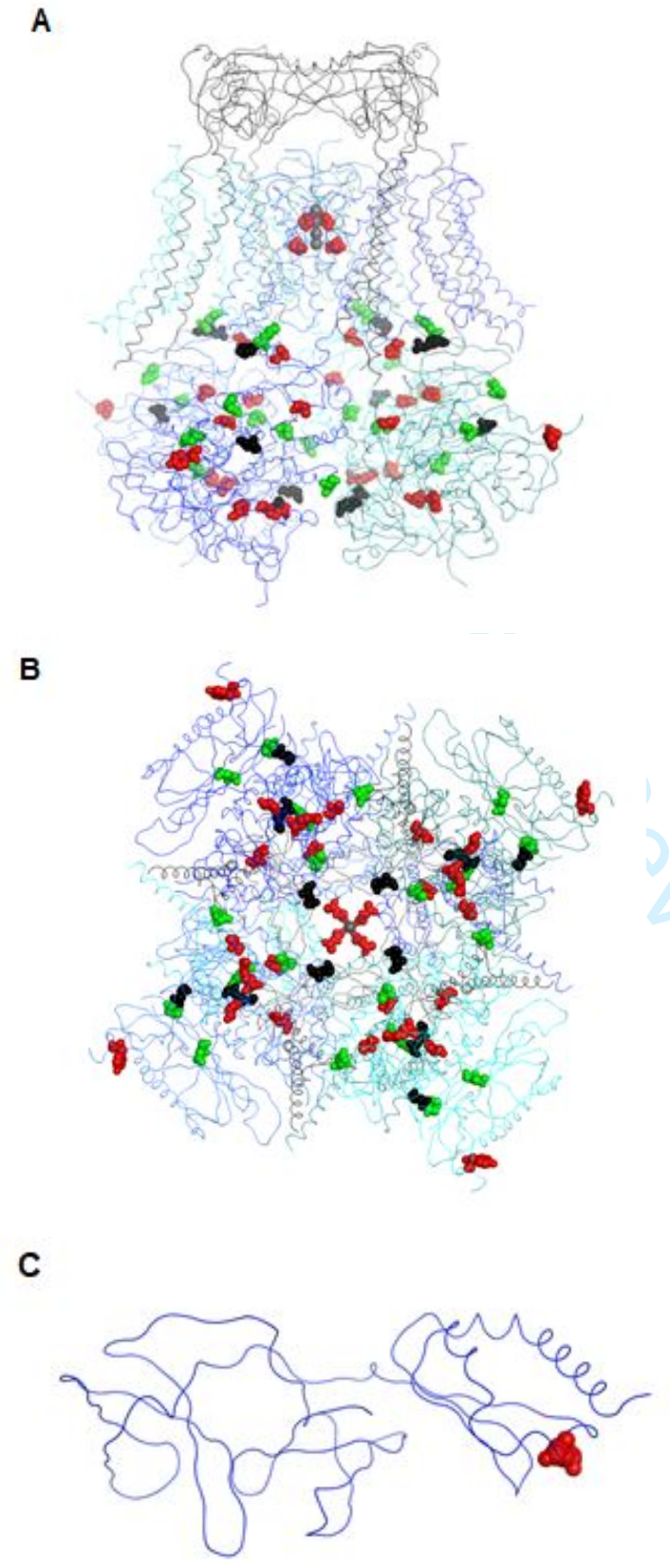

Figure 2. Cryo-EM structure of KCNMA1/KCNMB4 complex showing GoF (gain of function) and LoF (loss of function) variants/polymorphisms. (**A**) Side on view and (**B**) Top view looking from outside of plasma membrane, of channel with main chain only shown as a ribbon. The recently disclosed cryo-EM structure of the BK channel complexed with the ß4 subunit allowed us to map the BK channel variants (see Supplementary Table 1) onto the channel structure of the KCNMA1/KCNMB4 complex (hsSlo1-beta4, pdb 6v22).[19] This revealed that most variants were in the calcium sensing cytoplasmic domains of the tetramer though some loss of function (LoF) variants were present close to the K^+ channel itself. Most LoF polymorphisms and variants (red) were linked to intellectual disability while gain of function (GoF) changes (green) were more often linked to epilepsy and other movement disorders. Some of the reported changes have not been analysed using electrophysiology and these are shown in black. The structure of the N-terminal domain of FMRP was also reported together with the R138Q variant linked to a FXS like disorder. Mapping this variant onto the FMRP protein Colours KCNMA1 blue to cyan. KCBMB4 black. Variants shown as space-filling spheres, Red LoF, Green GoF, Black not determined. Coordinates taken from pdb 6v22. (**C**) FMRP protein showing the position of R138Q, coordinates from pdb 4qw2. Mapping this variant onto the FMRP protein (Figure 1C) revealed it to be in a highly accessible position of the molecule.[20] KCNMA1 variants were searched using the terms KCNMA1 and a citation search on the early paper.[11] A similar process was followed for the FMRP structure (pdb 4qw2). Only clinically relevant variants were included. The consensus sequence Q12791-1 was used. Many variants of the BK channel sequence appear in the literature and sometimes a given variant may have a different sequence number. The sequence context is shown in Supplementary Table 1 to avoid ambiguity.

Supplementary Table 1. Clinically relevant variants and polymorphisms in the BK channel that cause intellectual disability

Variant	Sequence context	Effect on channel	Clinical Phenotype	In structure	Reference and accession codes (where reported)
A138V	INNGSSQ**A**DG	Patient cells showed reduced activity of a K+ channel Small variable effect on currents in transfected HEK cells.	Severe ID, epilepsy, and autism spectrum disorder	no	Laumonnier 2006 Given as NM_002227 (possibly a misprint as this is Janus Kinase) probably NM_002247 Plante et al., 2019 MG279688
S351Y	ECVYLLMVTM **S**TVGYGDVYA	LoF abolished BK current.	Mild ID	yes	Liang et al., 2019 NM_002247.3
G354S	STV**G**YGDVYA	LoF When expressed by adenovirus in cerebellum rescued by NS1619.	ID with cerebellar ataxia Loss of mitochondrial function. Chlorzoxazone (BK/SK activator) gave partial improvement.	yes	Du et al., 2020 NM_002247
C413Y	VV**C**GHITLES	Partial LoF	mild to severe ID, speech delay, ataxia, axial hypotonia, and cerebral atrophy	yes	Liang et al., 2019 NM_002247.3
N449fs	VFLHNISP**N**L	Frame shift. LoF.	ID not reported but mild cerebellar atrophy	yes	Liang et al., 2019 NM_002247.3
N599D	KYYLEGVS**N**E MYTEYLSSAF	Possible LoF as does not facilitate opening	Not reported	yes	Plante et al., 2019 MG279688
I663V	HLKIQEGTLG FF**I**ASDAKEV	LoF abolishes BK current	ID, hypotonia, ataxia	yes	Liang et al., 2019 NM_002247.3
Y676L-fs	KRAFF**Y**CKAC	loss of function	GDD, epilepsy, severe cerebellar atrophy	yes	Tabarki et al., 2016. NM_001161352.1 Liang et al.,2019 NM_002247.3
R858W	SSALIGL**R**NL	LoF.		no	Plante et al., 2019 MG279688
P805L also described as P840L (P863L in	VM**P**LRASNFH	LoF, reduction in the amplitude of the BK current and	ID	yes	Bailey et al., 2020 NM_002247.3

reference sequence)		a shift to a positive potential for the activation curve			
D1008N listed as D984N in Liang 2019	IITELVN**D**TN	LoF, marked reduction in activation of the BK channel	Moderate ID	**yes**	Liang et al., 2019 NM_002247.3
D434G	KDR**D**DVNVEI	GoF	generalized epilepsy and paroxysmal dyskinesia	**yes**	Du et al., 2005 NM_002247
C495G	ANKY**C**ADPDA	GoF, shifts $V_{1=2}$ to more hyperpolarized potentials (by -15 to -20 mV)	Not reported	**yes**	Plante et al., 2019 MG279688
N536H	KAHLL**N**IPSW	GoF	ID, dystonia. Autism spectrum disorder.	**yes**	Zhang et al. 2020
K457E	ELEALF**K**RHF	Possible GoF as treatment of the patient with 3,4-diaminopyridine was successful.	Paroxysmal dyskinesia, ataxia.	**yes**	Buckley et al. 2020 NM_001161352.2
R458ter	ELEALFK**R**HF	Truncating variant. LoF AND GoF	ID, epilepsy, corticospinal-cerebellar tract atrophy, and paroxysmal dyskinesia.	**yes**	Yesil et al. 2018 NM_001161353
E884K	GSI**E**YLKREW	Not reported	GDD and paroxysmal non-kinesigenic dyskinesia	**yes**	Zhang Z. B. et al., 2015.
N1053S	YF**N**DNILTLI	Not reported	GDD and paroxysmal non-kinesigenic dyskinesia	**yes**	Zhang Z. B. et al., 2015. Moldenhauer 2020 MG279689.1 Du et al., 2005 NM_002247

Reference sequence used was Q12791-1 (NM_002247.4), ID, intellectual disability; LoF, loss of function; GoF, gain of function. N995S, N999S, and N1053S same amino acid substitution reported in the literature as at least three different reference sequencing number schemes, sequence context is shown for clarity.

Bailey CS, Moldenhauer HJ, Park SM, Keros S, Meredith AL. *KCNMA1*-linked channelopathy. J Gen Physiol. 2019 Oct 7;151(10):1173-1189.

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Plante AE, Lai MH, Lu J, Meredith AL. Effects of Single Nucleotide Polymorphisms in Human KCNMA1 on BK Current Properties. *Front Mol Neurosci*. 2019 Dec 3;12:285.

Tabarki B, AlMajhad N, AlHashem A, Shaheen R, Alkuraya FS. Homozygous KCNMA1 mutation as a cause of cerebellar atrophy, developmental delay and seizures. *Hum Genet*. 2016;135(11):1295-1298.

Yeşil G, Aralaşmak A, Akyüz E, *et al*. Expanding the Phenotype of Homozygous *KCNMA1* Mutations; Dyskinesia, Epilepsy, Intellectual Disability, Cerebellar and Corticospinal Tract Atrophy. *Balkan Med J*. 2018;35(4):336-339.

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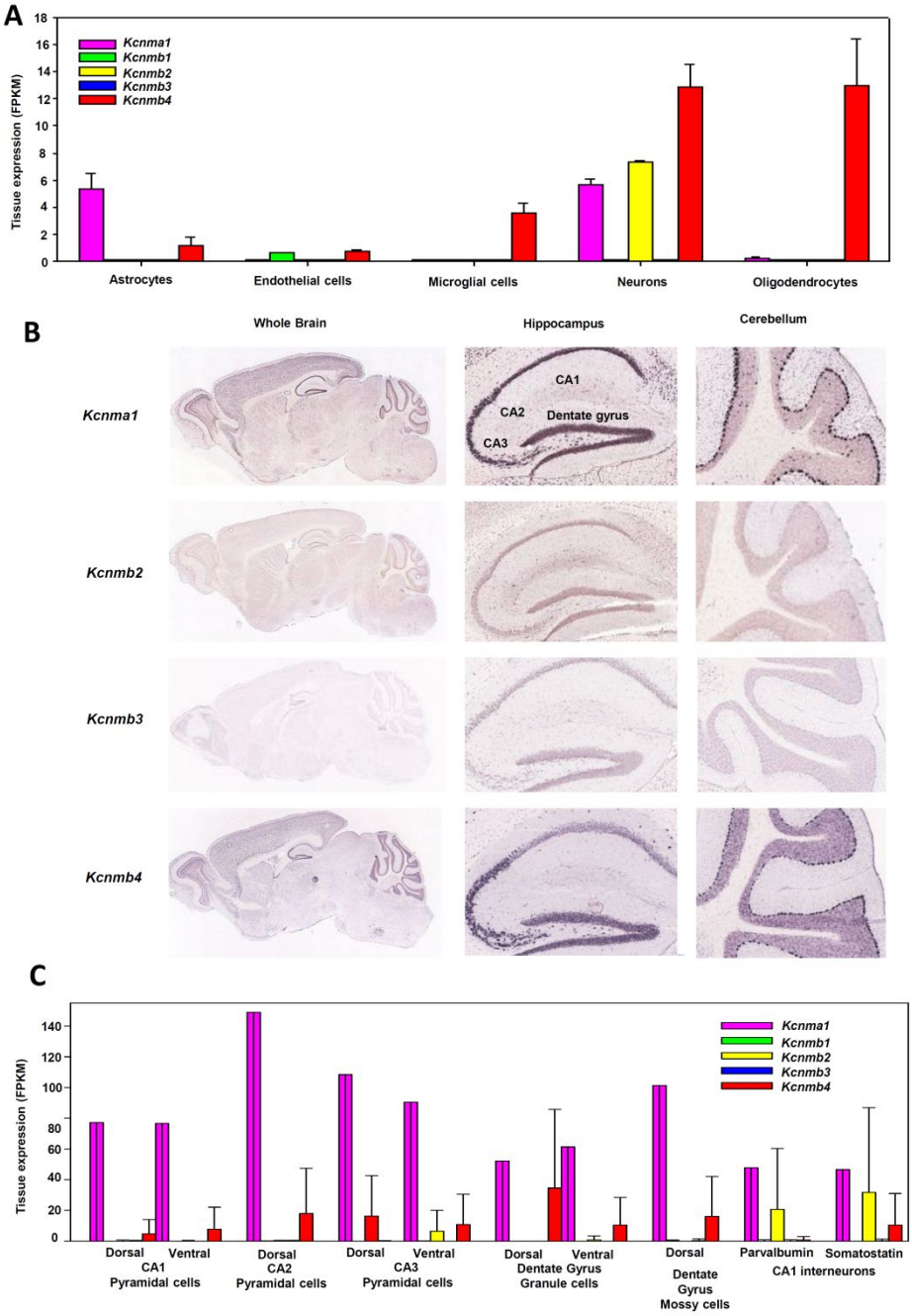
Supplementary Figure 1. Cellular expression of BK channel subunits in mouse brain

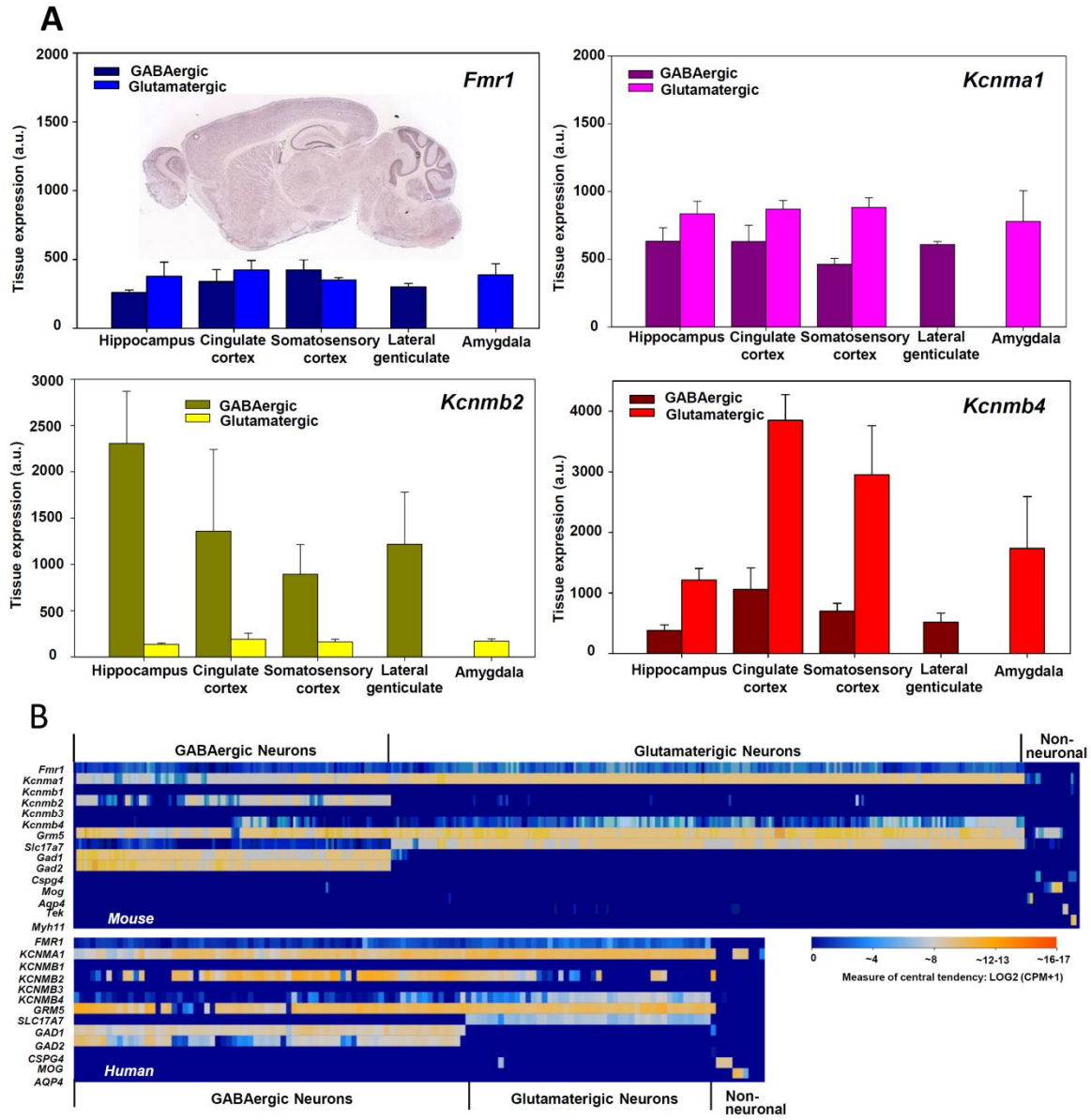


Supplementary Figure 1. Expression of BK isotypes in mouse brain. (**A**) RNAseq data was extracted from the BrainRNAseq portal for the BK isotypes. Results expressed as mean ± standard deviation for tissue expression as fragments per kilobase of transcript per million mapped reads (FPKM). (**B**) *In situ* hybridisation of BK isotypes in sagittal adult mouse brain sections reproduced from the Allen Mouse Brain Atlas showing expression of: *Kcnma1* (https://mouse.brain-map.org/experiment/show?id=74578206), *Kcnmb2* (https://mouse.brain-map.org/experiment/show?id=74635689), *Kcnmb3* (https://mouse.brain-map.org/experiment/show?id=81600586)

Kcnmb4 (https://mouse.brain-map.org/experiment/show?id=70593143)

(**C**) The expression BK subtypes in neuronal cells within the hippocampus. The results were extracted from the Hippocampus RNA-seq atlas and represent the mean and upper 95% confidence interval expressed as (FPKM) from 100 cells and 3 samples per group.

Supplementary Figure 2. Regional distribution and neuronal type of Fmr1 and BK channel subunits in mouse and human brain



Supplementary Figure 2. Differential expression of BK isotypes in glutamatergic and GABAergic neurons. RNAseq data was extracted from: (**A**) GEO profile. The results represent the mean and standard deviation of (n = 3-9/group) for the message for FMRP and the BK subtypes for glutamatergic and GABAergic neurons. The insert demonstrates *in situ* hybridization of *Fmr1* anti-sense in mouse brain extracted from the Allen brain Atlas (http://mouse.brain-map.org/experiment/show/73606703) (**B**) 10X RNAseq data from adult mouse cortex and hippocampus and human M1 cortex samples using the Allen Brain Map Transcriptomic explorer. The results represent a heatmap of cells grouped into transcriptomic cell types for: FMRP (*Fmr1*), BK channel subunits, metabotropic glutamate receptor 5 (*Grm5*),

vesicular glutamate transporter (*Slc17a7*) to detect glutamatergic cells, glutamic acid decarboxylase (*Gad*) one and two to detect GABAergic cells, NG2 chondroitin sulphate proteoglycan 4 (*Cspg4*) to detect oligodendrocyte precursors cells, myelin oligodendrocyte glycoprotein (*Mog*) to detect mature oligodendrocytes, aquaporin 4 (*Aqp4*) to detect astrocytes, endothelial tyrosine kinase (*Tek*) and myosin eleven (*Myh11*) to detect smooth muscle cells.

Supplementary Figures 1 and 2 narrative

Extraction of cell type specific RNAseq data (www.brainrnaseq.org) indicated that *Fmr1* message was notably expressed in mouse: astrocytes (58.9 ± 5.8 FPKM), neurons (13.8± 1.2 FPKM), endothelia (9.7± 0.2) and oligodendrocytes 3.4 ± 0.3 (FPKM) with low levels in microglia (1.3 ± 0.3). In rodents the *Kcnmb1* was largely restricted to the vasculature, consistent with known expression of KCNMB1 protein in smooth muscle within arteries (Baker et al. 2017. Figure 2A, Figure 3B). There was essentially no expression in neurons (Figure 2A, Figure 3B). Astrocytes expressed the BK channel alpha pore but there was limited astrocytic beta chain expression (Figure 2A, Figure 3B). These cells expressed the small conductance potassium channel KCNNR3 (www.brainrnaseq.org). Oligodendrocytes expressed KCNMB4 in rodents and humans (Figure 2A, Figure 3B), whereas oligodendrocyte precursors notably expressed KCNMA1 and KCNMB2 that was downregulated as they matured and developed KCNMB4 (Figure 3B). KCNMB3 was poorly expressed in the CNS (Figure 2A-C. Figure 3B).

As expected, the KCNMB4 isoform was associated with neural expression (Figure 2A-C, Figure 3A, B], although KCNMB2 was also present on neurons (Figure 2A-C, Figure 3A, B). *In situ* hybridisation demonstrated expression of *Kcnmb4* throughout the cortex and at high levels in the hippocampus and cerebellum (Figure 2B). This was confirmed in RNAseq data of the hippocampus with higher levels in the CA2 and CA3 regions (Figure 2C). In the hippocampus there was a low level of KCNMB2 expression except in the somatostatin (Sst) and parvalbumin (Pavalb) interneurons (Figure 2C). These were typically inhibitory, gamma aminobutyric acid (GABA) secreting neurons and suggested that KCNMB2 may be more prominent on GABAergic cells. Indeed, this was supported in microarray and RNAseq data in rodents (Figure 3A, 3B) and to some extent in humans (Figure 3B). *Fmr1* was detected throughout the brain (Figure 3A) on both GABAergic and glutamatergic neurons (Figure 3A), although some glutamatergic brain cells seemed to express high levels (Figure 3B). It appeared

that *Kcnmb4* and KCNMB4 was preferentially expressed by glutamatergic neurons in a variety of brain structures in mice and humans respectively (Figure 3A, 3B).

Microarray data from mouse neuronal forebrain (Sugino et al. 2006) was extracted from the Gene Expression Omnibus GDS1522 Accession number GSE2882. *In situ* hybridization data from mouse brain sections was extracted from the Allen Institute Brain Map (https://portal.brain-map.org). (Lein et al. 2007). RNAseq of mouse brain cell subtypes (www.brainrnaseq.org) (Zhang et al. 2014), mouse hippocampus RNAseq atlas (https://hipposeq.janelia.org) (Cembrowski et al. 2016) and 10X single cell RNAseq of mouse cortex and hippocampus (https://portal.brain-map.org/atlases-and-data/rnaseq/protocols-mouse-cortex-and-hippocampus) and human cortical cells (https://portal.brain-map.org/atlases-and-data/rnaseq/protocols-human-cortex) were extracted from the Allen Brain Map atlas (http://portal.brain-map.org). Supportive data was obtained from the Human protein atlas (www.proteinatlas.org Sjöstedt et al. 2020).

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Sjöstedt E, Zhong W, Fagerberg L, *et al*. An atlas of the protein-coding genes in the human, pig, and mouse brain. *Science*. 2020;367(6482) eaay5947.
Sugino K, Hempel CM, Miller MN, *et al*. Molecular taxonomy of major neuronal classes in the adult mouse forebrain. *Nat Neurosci*. 2006;9(1):99-107.
Zhang Y, Chen K, Sloan SA, *et al*. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci*. 2014;34:11929-47.

ARRIVE

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

Carol Kilkenny[1], William J Browne[2], Innes C Cuthill[3], Michael Emerson[4] and Douglas G Altman[5]

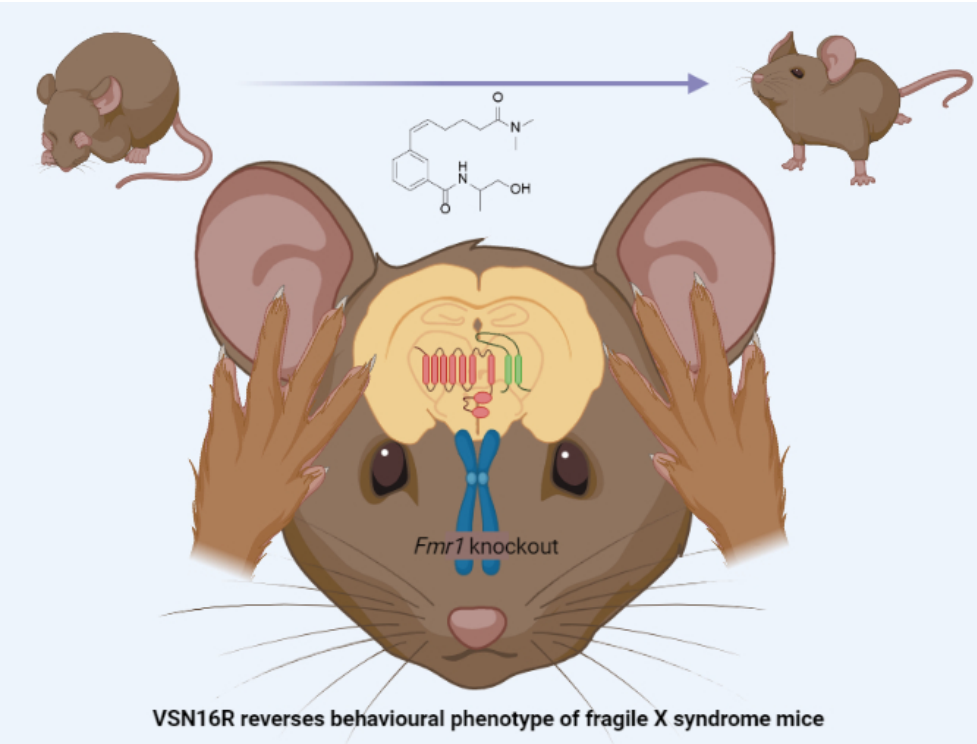
[1]The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, [2]School of Veterinary Science, University of Bristol, Bristol, UK, [3]School of Biological Sciences, University of Bristol, Bristol, UK, [4]National Heart and Lung Institute, Imperial College London, UK, [5]Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Title page
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Abstract page1
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	Introduction
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Introduction
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Methods Animals
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	Methods treatment groups
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	Methods behavioural tests
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Methods animals and treatment groups

The ARRIVE guidelines. Originally published in *PLoS Biology*, June 2010[1]

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	Methods animal housing
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	Methods treatment groups
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	Methods behavioural tests
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Methods behavioural
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	Methods data anlysis
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	Methods treatment tolerability
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	n = 10 per group
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Results behavioural
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	Methods treatment tolerability
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	Discussion
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Discussion
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	Acknowledgments

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.



VSN16R reverses behavioural phenotype of fragile X syndrome mice