REPORT

Reversal of behavioural phenotype by the cannabinoid-like compound VSN16R in fragile X syndrome mice

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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.

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Running title: VSN16R restores function in fragile X mice

Keywords: fragile X syndrome; VSN16R; BK channel; FMRP; *Fmr1* knockout mouse

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.

Acknowledgements

We would like to acknowledge FRAXA Research Foundation, USA for providing the mice for the behavioral studies and for supporting FRAXA-DVI, a FRAXA facility for drug development. Funding came from Fondecyt 120028. Additional funding came from the National MS Society – FastForward group (FF-1602-07939), the Wellcome Trust 092945, 206020/Z/16/Z, TSB/MRC 25036-166159, and Canbex Therapeutics. We are grateful to the curators of the Allen Brain Atlas, BrainRNAseq and Hipposeq for providing access to data.

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

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). 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.

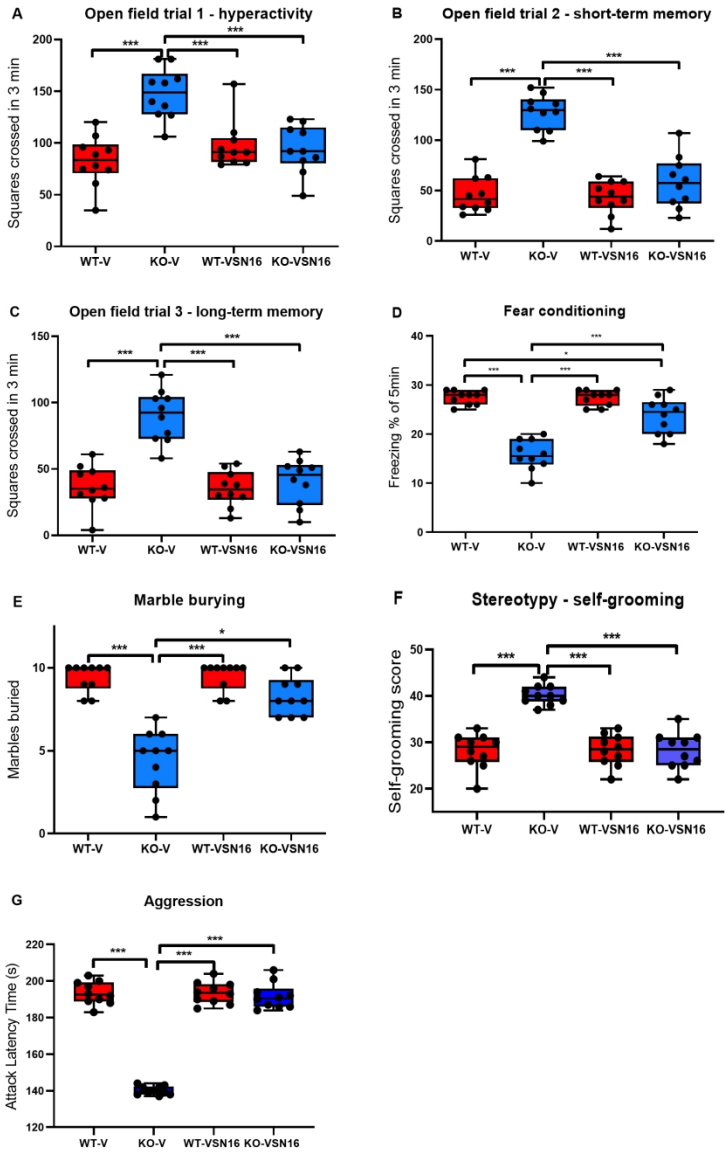

Figure 1

153x230mm (300 x 300 DPI)

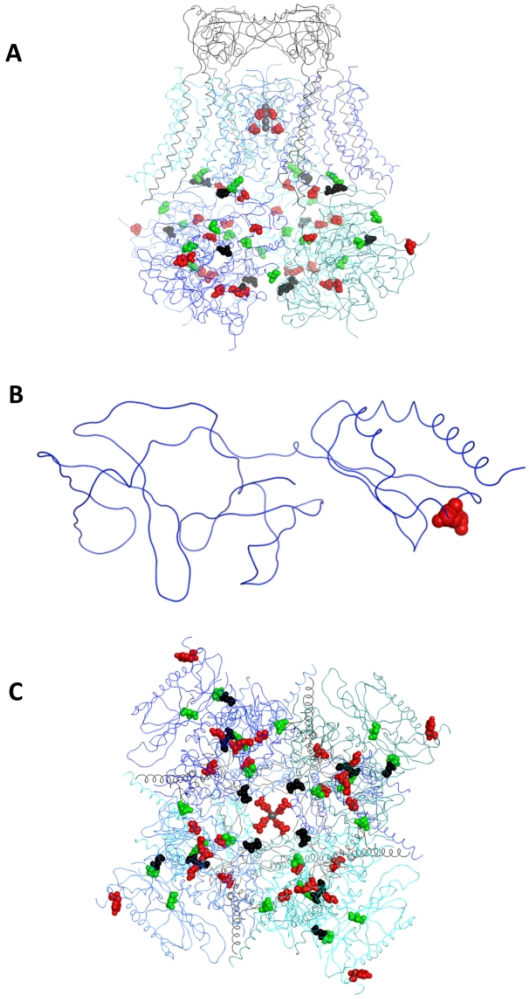

Figure 2

190x275mm (300 x 300 DPI)

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 reference	VM**P**LRASNFH	LoF, reduction in the amplitude of the BK current and a shift to a	ID	**yes**	Bailey et al., 2020 NM_002247.3

sequence)		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.

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