Functional Characterization of KCNMA1 mutation associated with dyskinesia, seizure, developmental delay, and cerebellar atrophy

Emrah Yucesan (emrahyucesan@yahoo.com)
Bezmialem Vakıf Üniversitesi https://orcid.org/0000-0003-4512-8764
Beyza Goncu
Bezmialem Vakif University: Bezmialem Vakif Universitesi
Cemil Ozgul
Istanbul Medipol University: Istanbul Medipol Universitesi
Arda Kebapci
Istanbul Medipol University: Istanbul Medipol Universitesi
Ayca Dilruba Aslanger
Istanbul University: Istanbul Universitesi
Enes Akyuz
Saglik Bilimleri Universitesi
Gozde Yesil
Istanbul University: Istanbul Universitesi

Research Article

Keywords: KCNMA1, gain-of-function, MCF7, immunostaining, patch-clamp

Posted Date: September 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-909709/v1

Abstract

KCNMA1 located on chromosome 10q22.3, encodes the pore-forming α subunit of the “Big K+” (BK) large conductance calcium and voltage-activated K + channel. BK channels are widely distributed across tissues, including both excitable and non excitable cells. Numerous evidence suggests the functional BK channel alterations produced by different *KCNMA1* alleles may associate with different symptoms, such as paroxysmal non kinesigenic dyskinesia with gain of function and ataxia with loss of function. Functional classifications revealed two major patterns, gain of function and loss of function effects on channel properties in different cell lines. In the literature, two mutations have been shown to confer gain of function properties to BK channels: D434G and N995S. On the other hand 10 mutations have been classified as loss of function (S351Y,G354S, G356R, G375R, C413Y/N449fs, I663V, P805L, and D984N) or putative loss of function (premature truncation mutations: Y676Lfs*7 and Arg458Ter). In this study, we report the functional characterization of a variant which was previously reported the whole exome sequencing revealed bi-allelic nonsense variation (NM_001161352.1 (ENST00000286628.8):c.1372C > T; Arg458*) of the cytoplasmic domain of calcium-activated potassium channel subunit alpha-1 protein. To detect functional consequences of the variation immunostaining and electrophysiological studies were conducted. In this study, we conducted patch-clamp recordings on WT and R458X mutant cells. We found the gain of function effect for the mutation. This is the first functional study observing an increased current in the *KCNMA1* gene resulting from a truncating mutation

Introduction

The potassium calcium-activated channel subfamily m alpha 1 (*KCNMA1*) gene encodes the alpha subunit of the large conductance, voltage, and calcium-sensitive potassium channel (BK channels) that plays a critical role in neuronal excitability (Yesil et al. 2018). The *KCNMA1*, located on chromosome 10q22.3, encodes the α-subunit of the BK channel (Dworetzky et al. 1994). This α-subunit contains seven transmembrane domains, named as S0-S6 at N-terminus and a large intracellular C-terminus (Liang et al. 2019). In the literature, several studies were reported on every segment for N-terminus (Yan and Aldrich 2010, 2012; Yang et al. 2015) and also for C-terminus (Lee and Cui 2010; Yuan et al. 2010; Wang and Sigworth 2009). The comprehensive structure of BK channels was determined by Yuan P. et al. (Yuan et al. 2011). Mutations in the *KCNMA1* gene cause neurological phenotypes such as susceptibility to generalized epilepsy (MIM: 618596), cerebellar atrophy, developmental delay, seizures (MIM: 617643), Liang-Wang syndrome (MIM: 618729), and paroxysmal nonkinesigenic dyskinesia, 3 (MIM: 609446). Among these diseases overlapping phenotypes have been reported and inheritance pattern differences vary, from autosomal dominant to autosomal recessive.

Herein, we present the functional characterization of R458X variation in *KCNMA1* (NM_001161352.1:c.1372C > T,p.Arg458*) that our team had previously identified a patient who presented with a mixed phenotype with nonkinesigenic dyskinesia, cerebellar atrophy, and epilepsy showing both gain and loss of function (Yesil et al. 2018). To demonstrate the functional effect of the variant a two step-approach was conducted; on the one hand, immunostaining was carried out to show

the subcellular effect of the protein level, and on the other hand, patch-clamp technique was accomplished to determine how the membrane conductance differs between mutant vs. wild-type protein. In this way. NM_001161352.1:c.1372C > T,p.Arg458* variation was considered as a causative mutation for the patient's phenotype. To our knowledge, this is the first truncating mutation in *KCNMA1* gene that has a gain of function potential. We have demonstrated the importance of the BK channels and the relationship between changes in their functions, both for gain or loss of function, and the disease.

Materials And Methods

Genetic analysis and functional characterization

In this study, we report the functional characterization of a variant which was previously reported (Yesil et al. 2018). The detected variant was evaluated using a proper in-silico prediction tool to confirm previous findings and control update variations. Population cohort databases were also searched in terms of the variant. To detect functional consequences of the variation immunostaining and electrophysiological studies were conducted, respectively. For this purpose, the eGFP-tagged wild type and mutant type *KCNMA1*-expressing plasmids were obtained from Gene Universal (Delaware, USA). Then the transfection process started.

Transfection process

MCF7 (Michigan Cancer Foundation 7) cells (ATCC® HTB-22™) were used for functional assessment. The cells were grown in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Whole cultivation supplies were purchased from PAN-Biotech (Aidenbach, Germany). Cells were cultivated in a humidified atmosphere with 5% CO_2 at 37 °C. In a typical 96-well plate, the transfection efficiency was optimized. In this experiment; 5×10^3 cells/well MCF7 cells were transfected in Opti-MEM by the addition of Lipofectamine 2000 (ThermoFisher Scientific, MA, USA) containing 200 and 400 ng of each eGFP-tagged-pDNA encoding both wild and mutant type separately from *KCNMA1*. The lipofectamine and pDNA ratio was 1:1. After 24 hours of cultivation, the medium was removed and the wells were washed with 1X PBS then the efficiency was compared macroscopically between different pDNA groups. Determining concentrations of pDNA were further used for confocal microscopy and patch-clamp experiments, respectively. All experiments were performed in triplicates.

Laser scanning confocal microscopy

The two-well chamber slide was used for confocal microscopy. 10×10^5 cells were seeded in each well. Transfection was performed with Lipofectamine 2000 including 400 ng pDNA/well for wild and mutant type eGFP-tagged-KCNMA1. After transfection for 24 hours, MCF7 cells were incubated with 0.01 mg/mL DAPI at room temperature for 15 minutes. After this, the fluorescence signal of cells was assessed by laser scanning confocal microscopy (Leica TCS-SPE, Leica Microsystems, Wetzlar, Germany) at Aziz Sancar Institute of Experimental Medicine of Istanbul University. Three images were analyzed for each

wild type and mutant group by ImageJ (National Institutes of Health, Bethesda, MD) (Schneider et al. 2012).

Patch-clamp

Membrane currents were recorded using the whole-cell configuration of the patch-clamp method. For the electrophysiological recordings of the selective potassium channel, 140 mM NaCl extracellular bath solution was prepared as 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 15 mM glucose (pH: 7.3 with NaOH), while the intracellular pipette solution was 130 mM KCl, 0.5 mM $MgCl_2$, 10 mM HEPES, and 5 mM EGTA (pH: 7.2 with KOH). Also, 3-5 MΩ pipette resistance was used. The recordings were taken using the voltage-clamp method with a whole cell configuration. Holding potential was fixed at -60 mV and voltage from -100 mV to +50 mV (step by step 10 mV) was applied for 500 ms and current responses were measured (Axon Digidata 1550, Axon Multiclamp 700B). Recordings were made at room temperature.

Results

As previously reported, the whole exome sequencing revealed bi-allelic nonsense variation (NM_001161352.1 (ENST00000286628.8):c.1372C > T; Arg458*) of the cytoplasmic domain of calcium-activated potassium channel subunit alpha-1 protein (Yesil et al. 2018)

This variation has not been reported in population and mutation databases and is predicted to have a deleterious effect according to prediction databases such as mutation taster, DANN, and FATHMM-MKL. According to ACMG classifications, this variant is classified as PVS1 pathogenic since it is a null variant where the gene *KCNMA1* has a high LOF Z score (6.08) and seemed to be located in a highly conserved locus throughout different species with a PhloP score of (phylogenetic p-values) 2.672 (Kopanos et al. 2019).

Immunostaining was carried out to compare the expression level between wild-type and mutant cells. For this aim, 400 ng pDNA was transfected and the average transfection efficiency was found as 70% for the wild type and 62% for the mutant type (Fig. 1). MCF7 cell growth rate was reduced after mutant type KCNMA1 transfection however this outcome was not evaluated specifically as this was not a primary research question for this study. We have detected the decreased proliferation rate when we compared the mutant type with the wild-type (Fig. 1). Later, patch-clamp recordings were taken in triplicate from MCF-7 cells for both wild-type and mutant/variant cells in standard conditions as mentioned above and representative current densities are shown in Fig. 2. Outward currents of X channel mutant cells were found significantly higher than wild type groups ($p = 0.0005$).

Discussion

The presence of different currents in various tissues affects the complex role of BK channels in the regulation of excitability (Bailey et al. 2019). Therefore, defining the mutation's functional

characterization seems mandatory. Numerous phenotypes were associated with *KCNMA1* mutations. Particularly epilepsy, movement disorders, developmental delay, and cerebellar atrophy have been reported (Du et al. 2005; Zhang et al. 2015; Li et al. 2018; Tabarki et al. 2016). In the context of genetic heterogeneity, some of these variations show different BK current properties. BK channels play an important role in neuronal excitability via balancing potassium and calcium potential from depolarization to hyperpolarization. However, the exact mechanisms by which KCNMA1 variations effects BK channel activity remain unclear (Bailey et al. 2019). The understanding mechanistic explanation of the BK channel features and activities are not only crucial for neurological systems, but also muscular and osteoblastic activity (Jaggar et al. 2000; Tricarico et al. 2017; Hei et al. 2016).

As previously accounted, BK channel activity decreases the excitation feature of synaptic activity at neuromuscular junctions (Robitaille et al. 1993). Interestingly BK channels have a dual effect on neurons both for excitation and inhibition. Therefore, finding BK current activity shows different expression patterns for suppression to induction. For instance, Du et al. showed a missense variation, Asp434Gly in the regulator of conductance for K+ (RCK) domain in *KCNMA1*. In their study, Du et al. evaluated a large family with 13 individuals. Among them, one patient had only epilepsy, seven patients had paroxysmal dyskinesia and five patients had epilepsy with paroxysmal dyskinesia. Detected variations exhibited autosomal dominant inheritance patterns (Du et al. 2005). Latterly, the same mutation was detected and analyzed with different materials by other study groups (Diez-Sampedro et al. 2006; Wang et al. 2009; Yang et al. 2010). They demonstrate that the variation causes increased current activity, and they speculated a potential mechanism to account for disease in terms of this very specific variation. Briefly, this mechanism is G-V shift to hyperpolarized potentials, increased open probability, faster activation, slower deactivation, and increased Ca^{2+} sensitivity (Bailey et al. 2019). In contrast to these findings, Liang et al. reported three unrelated patients who had clinical heterogeneity, i.e. multiple malformation syndromes, such as facial abnormalities, global developmental delay, axial hypotonia, and visceral malformations. Also, one patient had a movement disorder, and two of the three had epileptic seizures (Liang et al. 2019). Similar to R458X reported by Yesil et.al, the variations C413Y, N449fs, and I663V reported by Liang et al. are placed in the RCK1 domain which is a binding site for Ca^{2+} (Liang et al. 2019). The clinical comparison of the patient carrying biallelic mutations C413Y/N449fs presented in and the patient presented by Yesil et. al. had developmental/intellectual deficiency and cerebellar ataxia in common however the patient reported by Yesil et. al had also dyskinesia and epilepsy phenotype (Yesil et al. 2018). Liang et al. observed that C413Y is a LoF variant that inhibits the function of the BK channel substantially and likely reduces the activation and macroscopic current amplitude of the BK channel significantly at calcium concentrations ranging from nominal 0 μM–10 Mm. Moreover, the frameshift variant p.(Asn449fs) did not elicit any potassium current under the voltage stimulus from − 160 mV–160 mV at a 10 μM calcium concentration suggesting that it is also a LoF variant.

In this study report, we conducted patch-clamp recordings on WT and R458X mutant cells. To understand how a truncating mutation abolishes function and the electrophysiological outcome, a patch-clamp with cell-attached recordings is a direct method. We measured current density from the ratio of peak current

amplitude to the cell membrane capacitance (picoamperes per picofarad (pA/pF)) under a stable pH. We found out that under a stable pH the mutant type showed an increasing sequence of actions when compared to the wild type. While the stimulus continued, changes in the potassium channel activation might have altered downstream potential as well. It seems that the increased current and thus the hyperpolarized state -of the channel firstly alters the function but negatively affects the viability of the cell later on (Fig. 1, 2). The dual phenotype with epilepsy/dyskinesia and cerebellar atrophy, may be explained with this mechanism in such severe gain of function mutations. Although gain-of-function have been generally shown in missense mutations nonsense mutations with a gain of function consequence have also been reported in the literature (Ho et al. 2007; Kuehnet al. 2017).

Taken together, the nature of the BK-channel and its effect on organisms, show a giant heterogeneity, from transcript variation to cellular electrical activity. Both the literature and this study have shown that the, changes in the RCK domain of BK channels directly affect protein function (Du et al. 2005; Zhang et al. 2010; Kim et al. 2008). This is the first functional study observing an increased current in the *KCNMA1* gene resulting from a truncating mutation. In conclusion, due to its complexity of their nature every causative variant in the channel genes should be evaluated in terms of functional characterization, particularly electrophysiological analyzes.

Declarations

Acknowledgements

This work was supported by the grants of Scientific Research Projects Coordination Unit of Bezmialem Vakif University (Project #2.2019/7). We highly appreciate the efforts of Monica Ozkan, MSN, RN, and CPAN in language editing of this paper. We also thank Sevde Hasanoglu for technical support with the equipment setup

References

1. Bailey CS, Moldenhauer HJ, Park SM, Keros S, Meredith AL (2019) KCNMA1-linked channelopathy. J Gen Physiol Oct 7(10):1173–1189. doi:10.1085/jgp.201912457 151) .
2. Diez-Sampedro A, Silverman WR, Bautista JF, Richerson GB (2006) Mechanism of increased open probability by a mutation of the BK channel. J Neurophysiol Sep 96(3):1507–1516. doi:10.1152/jn.00461.2006
3. Du W, Bautista JF, Yang H et al (2005) Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. Nat Genet Jul 37(7):733–738. doi:10.1038/ng1585
4. Dworetzky SI, Trojnacki JT, Gribkoff VK (1994) Cloning and expression of a human large-conductance calcium-activated potassium channel. Brain Res Mol Brain Res Nov 27(1):189–193. doi:10.1016/0169-328x(94)90203-8

5. Hei H, Gao J, Dong J et al (2016) BK Knockout by TALEN-Mediated Gene Targeting in Osteoblasts: KCNMA1 Determines the Proliferation and Differentiation of Osteoblasts. Mol Cells Jul 39(7):530–535. doi:10.14348/molcells.2016.0033
6. Ho MS, Tsang KY, Lo RL et al (2007) COL10A1 nonsense and frame-shift mutations have a gain-of-function effect on the growth plate in human and mouse metaphyseal chondrodysplasia type Schmid. Hum Mol Genet May 15(10):1201–1215. doi:10.1093/hmg/ddm067 16) .
7. Jaggar JH, Porter VA, Lederer WJ, Nelson MT (2000) Calcium sparks in smooth muscle. Am J Physiol Cell Physiol Feb 278(2):C235–C256. doi:10.1152/ajpcell.2000.278.2.C235
8. Kim HJ, Lim HH, Rho SH et al (2008) Modulation of the conductance-voltage relationship of the BK Ca channel by mutations at the putative flexible interface between two RCK domains. Biophys J Jan 15(2):446–456. doi:10.1529/biophysj.107.108738 94) .
9. Kopanos C, Tsiolkas V, Kouris A et al (2019) VarSome: the human genomic variant search engine. Bioinformatics Jun 1 35(11):1978–1980. doi:10.1093/bioinformatics/bty897
10. Kuehn HS, Niemela JE, Sreedhara K et al (2017) Novel nonsense gain-of-function NFKB2 mutations associated with a combined immunodeficiency phenotype. Blood Sep 28(13):1553–1564. doi:10.1182/blood-2017-05-782177 130) .
11. Lee US, Cui J (2010) BK channel activation: structural and functional insights. Trends Neurosci Sep 33(9):415–423. doi:10.1016/j.tins.2010.06.004
12. Li X, Poschmann S, Chen Q et al (2018) De novo BK channel variant causes epilepsy by affecting voltage gating but not Ca(2+) sensitivity. Eur J Hum Genet Feb 26(2):220–229. doi:10.1038/s41431-017-0073-3
13. Liang L, Li X, Moutton S et al (2019) De novo loss-of-function KCNMA1 variants are associated with a new multiple malformation syndrome and a broad spectrum of developmental and neurological phenotypes. Hum Mol Genet Sep 1(17):2937–2951. doi:10.1093/hmg/ddz117 28) .
14. Robitaille R, Adler EM, Charlton MP (1993) Calcium channels and calcium-gated potassium channels at the frog neuromuscular junction. J Physiol Paris 87(1):15–24. doi:10.1016/0928-4257(93)90020-t
15. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods Jul 9(7):671–675
16. Tabarki B, AlMajhad N, AlHashem A, Shaheen R, Alkuraya FS (2016) Homozygous KCNMA1 mutation as a cause of cerebellar atrophy, developmental delay and seizures. Hum Genet Nov 135(11):1295–1298. doi:10.1007/s00439-016-1726-y
17. Tricarico D, Mele A. Commentary (2017) A BK (Slo1) channel journey from molecule to physiology. Front Pharmacol 8:188. doi:10.3389/fphar.2017.00188
18. Wang B, Rothberg BS, Brenner R (2009) Mechanism of increased BK channel activation from a channel mutation that causes epilepsy. J Gen Physiol Mar 133(3):283–294. doi:10.1085/jgp.200810141
19. Wang L, Sigworth FJ (2009) Structure of the BK potassium channel in a lipid membrane from electron cryomicroscopy. Nature Sep 10(7261):292–295. doi:10.1038/nature08291 461) .

20. Yan J, Aldrich RW (2010) LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium. Nature Jul 22(7305):513–516. doi:10.1038/nature09162 466) .
21. Yan J, Aldrich RW (2012) BK potassium channel modulation by leucine-rich repeat-containing proteins. Proc Natl Acad Sci U S A May 15(20):7917–7922. doi:10.1073/pnas.1205435109 109) .
22. Yang H, Zhang G, Cui J (2015) BK channels: multiple sensors, one activation gate. Front Physiol 6:29. doi:10.3389/fphys.2015.00029
23. Yang J, Krishnamoorthy G, Saxena A et al (2010) An epilepsy/dyskinesia-associated mutation enhances BK channel activation by potentiating. Ca2 + sensing Neuron Jun 24(6):871–883. doi:10.1016/j.neuron.2010.05.009 66) .
24. Yesil G, Aralasmak A, Akyuz E, Icagasioglu D, Uygur Sahin T, Bayram Y (2018) Expanding the Phenotype of Homozygous KCNMA1 Mutations; Dyskinesia, Epilepsy, Intellectual Disability, Cerebellar and Corticospinal Tract Atrophy. Balkan Med J Jul 24(4):336–339. doi:10.4274/balkanmedj.2017.0986 35) .
25. Yuan P, Leonetti MD, Pico AR, Hsiung Y, MacKinnon R (2010) Structure of the human BK channel Ca2+-activation apparatus at 3.0 A resolution. Science Jul 9(5988):182–186. doi:10.1126/science.1190414 329) .
26. Yuan P, Leonetti MD, Hsiung Y, MacKinnon R (2011) Open structure of the Ca2 + gating ring in the high-conductance Ca2+-activated K + channel. Nature Dec 4(7379):94–97. doi:10.1038/nature10670 481) .
27. Zhang G, Huang SY, Yang J et al (2010) Ion sensing in the RCK1 domain of BK channels. Proc Natl Acad Sci U S A Oct 26(43):18700–18705. doi:10.1073/pnas.1010124107 107) .
28. Zhang ZB, Tian MQ, Gao K, Jiang YW, Wu Y (2015) De novo KCNMA1 mutations in children with early-onset paroxysmal dyskinesia and developmental delay. Mov Disord Aug 30(9):1290–1292. doi:10.1002/mds.26216

Figures

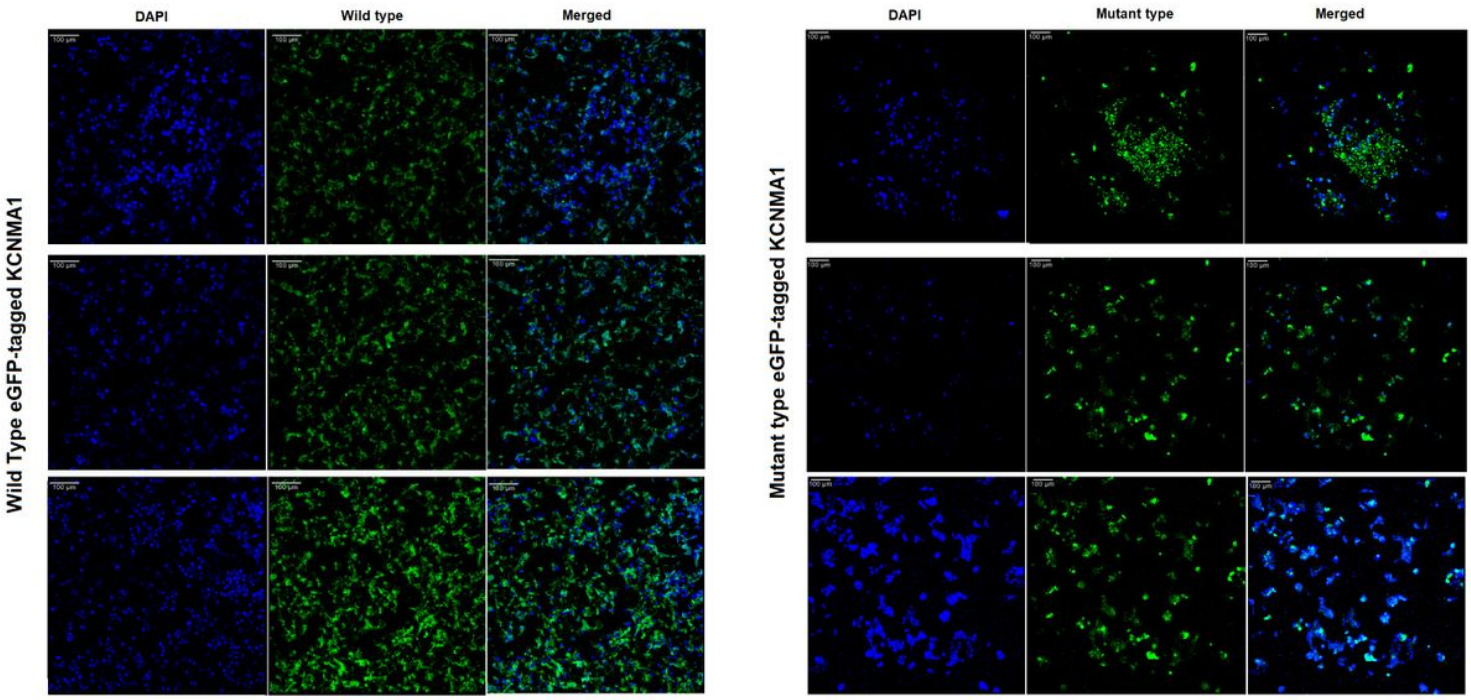

Figure 1

Transfection efficiency results in MCF-7 cells for both wild and mutant type eGFP-tagged-KCNMA1. The nuclei were stained with DAPI in blue and transfected cells are in green. Reduced proliferation were observed cells expressing mutant type KCNMA1 when compared with wild type. Results were demonstrated in triplicates. Images were taken using confocal laser scanning microscopy.

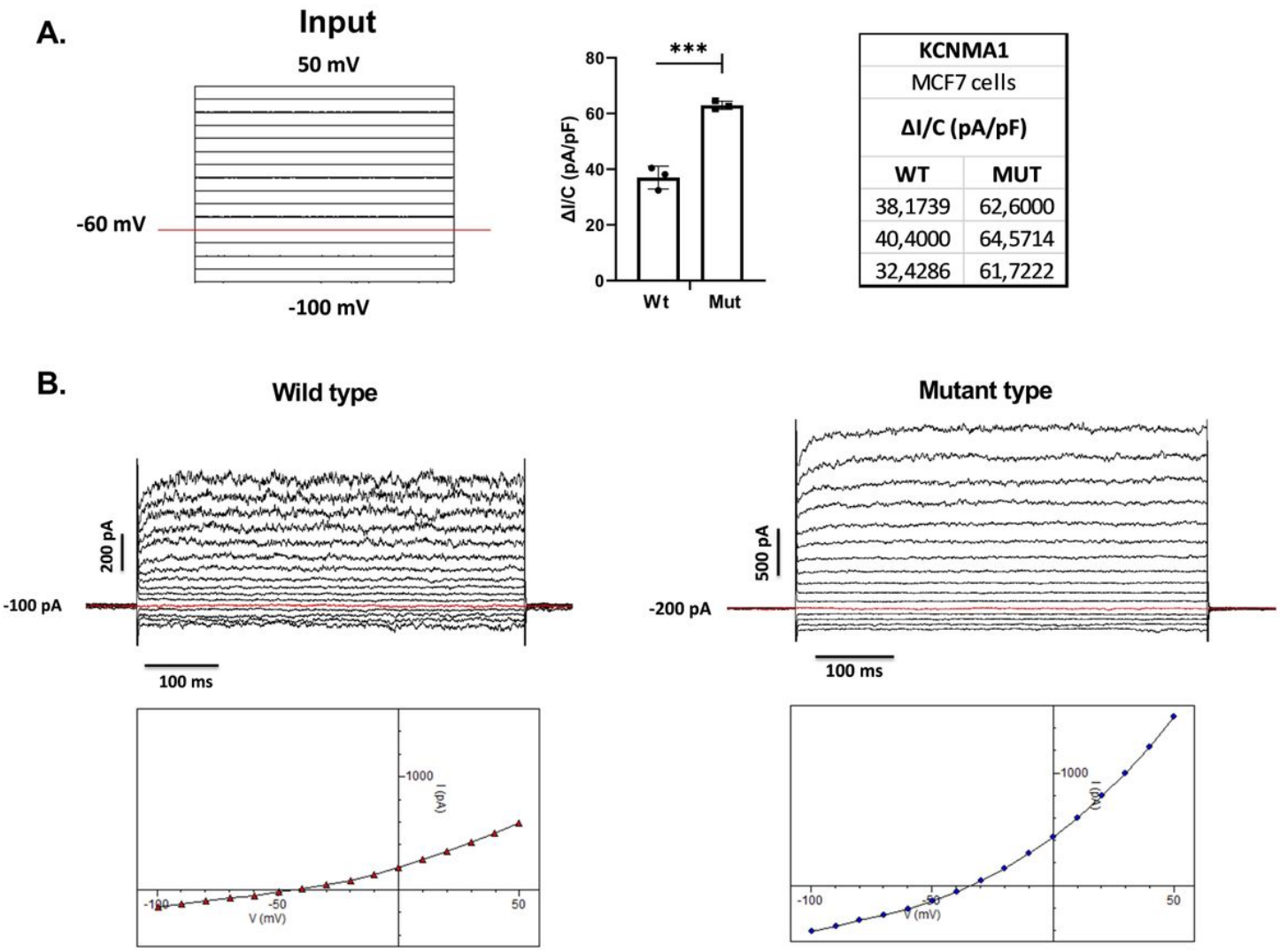

Figure 2

Patch-clamp recordings from MCF7 cells. A. Shows voltage protocol, statistical analyze of the pA/pF ratio and raw data of peak current amplitude to the cell membrane capacitance respectively. Statistical analyzis performed with a t-test (p=0.0005). B. Whole cell patch-clamp recordings of wild and mutant type respectively. pA/pF: picoamperes per picofarad.