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SCN4A mutation as modifying factor of Myotonic Dystrophy Type 2 phenotype

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ABSTRACT

In myotonic dystrophy type 2 (DM2), an association has been reported between early and severe myotonia and recessive chloride channel (CLCN1) mutations. No DM2 cases have been described with sodium channel gene (SCN4A) mutations. The aim is to describe a DM2 patient with severe and early onset myotonia and co-occurrence of a novel missense mutation in SNC4A. A 26-year-old patient complaining of hand cramps and difficulty relaxing her hands after activity was evaluated at our department. Neurophysiology and genetic analysis for DM1, DM2, CLCN1 and SCN4A mutations were performed. Genetic testing was positive for DM2 (2650 CCTG repeat) and for a variant c.215C>T (p.Pro72Leu) in the SCN4A gene. The variation affects the cytoplasmic N terminus domain of Nav1.4, where mutations have never been reported. The biophysical properties of the mutant Nav1.4 channels were evaluated by whole-cell voltage-clamp analysis of heterologously expressed mutant channel in tsA201 cells. Electrophysiological studies of the P72L variant showed a hyperpolarizing shift (−5 mV) of the voltage dependence of activation that may increase cell excitability. This case suggests that SCN4A mutations may enhance the myotonic phenotype of DM2 patients and should be screened for atypical cases with severe myotonia.

Keywords: Myotonic dystrophy type 2; Myotonia; SCN4A; N-terminus

1. Introduction

Myotonic dystrophy type 2 (DM2, PROMM, OMIM # 602668) is an adult onset muscular dystrophy caused by a CCTG repeat expansion in CNBP gene on chromosome 3q21 [1]. The expanded RNA transcripts modify the activity of specific RNA binding proteins involved in regulating alternative splicing. The resulting missplicing of several genes is thought to account for the multisystem nature of the disease including cardiac, cerebral and endocrine involvement, along with proximal weakness and myotonia [2]. Missplicing of the skeletal muscle chloride channel, CLC-1, leads to a transcript coding for a non-functional channel. Consequent reduced resting

chloride conductance increases the electrical excitability of the muscle and causes myotonia [3]. Myotonia in DM2 is usually mild and sometimes may be difficult to elicit even with electromyographic evaluation [4], [5]. Recent studies have found an association between DM2 patients with prominent myotonia and heterozygous recessive CLCN1 mutations on chromosome 7q35 [6], [7]. Mutations of CLCN1 which encode the skeletal muscle voltage-gated chloride channel (CLC-1) are responsible for myotonia congenita (recessive Becker disease, OMIM # 255700; dominant Thomsen disease, OMIM # 160800). Mutations are "loss of function" causing reduced sarcolemmal chloride conductance and enhanced excitability [8]. The additive effects of CLCN1 missplicing caused by DM2 expansion and CLCN1 mutation may cause an atypical DM2 phenotype characterized by severe and early myotonia [7].

Another gene implicated in myotonic disorders is SCN4A on chromosome 17q23.3 (Myotonia, Potassium-aggravated, OMIM # 608390). SCN4A codes for Nav1.4, the voltage gated sodium channel (VGSC) expressed in skeletal muscle [9]. SCN4A mutations usually produce a "gain of function" effect causing impaired inactivation or enhanced activation of the Nav1.4 channel resulting in muscle excitability [8]. To date, a possible additive influence of SCN4A mutation to the DM2 phenotype has never been evaluated.

In our study we investigated a DM2 patient with severe and early onset myotonia without mutation in CLCN1 gene. We identified a novel missense mutation c.215C>T (p.Pro72Leu) in SCN4A gene that represents the first mutation ever reported affecting the cytoplasmic N terminus domain of Nav1.4. To investigate the biophysical alteration of P72L substitution we performed whole-cell voltage clamping in a heterologous expression system showing the Nav1.4 mutant gain of function effect.

2. Materials and Methods

2.1. Patient. The patient, a Caucasian 26-year-old woman, was evaluated in the Department of Neurology at the Penn State Hershey Medical Center. The patient gave written informed consent for

genetic analysis in agreement with Helsinki convention. Clinical information on other family members was obtained during the interview.

2.2. Molecular genetic analysis. Genetic analysis of DMPK, CNBP, CLCN1 and SCN4A genes was performed by Athena Diagnostics, Inc. (Marlborough, MA) using the Complete Myotonia evaluation kit, which has been developed for the molecular diagnosis of myotonic dystrophy type 1 (DM1), myotonic dystrophy type 2 (DM2), myotonia congenita and sodium channel myotonia.

DM1 and DM2 repeat expansion mutations on DMPK gene (Chr19q13.3) and CNBP gene (Chr3q21.3) were evaluated utilizing polymerase chain reaction (PCR) amplification of genomic DNA followed by high resolution electrophoresis to determine the number and the size range of CTG and CCTG repeats.

DNA sequence analysis of CLCN1 and SCN4A gene was performed by PCR amplification of highly purified genomic DNA, followed by automated bi-directional DNA sequencing of the entire coding region of SCN4A (24 exons, NM_000334) and CLCN1 (23 exons, NM_000083). Sequencing also included the highly conserved flanking intronic sequence of the exon–intron splice junctions for all coding exons and 10 bases of intronic DNA surrounding each exon. Additional information is available by contacting Athena Diagnostic, Inc. SCN4A exon 1 has also been analyzed by direct sequencing in 100 control individuals.

2.3. Functional analysis of WT and p. P72L Nav 1.4 channels. The c.215C>T mutation was engineered into Wild-Type (WT) SCN4A complementary DNA (cDNA), received from Maria Essers Department of Clinical Research Ion Channels and Channelopathies University of Bern in the pRc/CMV2 plasmid by site directed mutagenesis using the QuikChange II site-directed mutagenesis kit (Stratagene). The construct was sequenced to confirm the correct introduction of mutation and ensure the validity of sequence. Beta 1 subunit (a kind gift from Prof. Hugues Abriel, University of Bern, Switzerland) was subcloned in a pIRES vector engineered with EGFP. WT and

P72L SCN4A mutant channels were transiently expressed in tsA201 cells using Fugene. An equal amount (0.5 µg) of alpha and beta 1 subunits were transfected. To mimic the heterozygous condition, 0.25 µg of each alpha subunit were transfected. The expression of the channels was studied 48 h after transfection.

Membrane currents were measured using whole cell patch clamp with Axopatch Multiclamp 700B amplifier (Axon Instruments, Foster City, CA), as previously described [10]. Recordings were made at room temperature. Internal solution contained (mmol/L) 50 aspartic acid, 60 CsCl, 5 disodium ATP, 11 EGTA, 10 HEPES, 1 CaCl2, 1 MgCl2, pH 7.4 adjusted with CsOH. Extracellular solution contained (mmol/L) 130 NaCl, 2 CaCl2, 5 CsCl, 1.2 MgCl2, 10 HEPES, 5 Glucose, pH 7.4 adjusted with CsOH. In the experiments designed to measure the voltage dependence of activation, external sodium was reduced to 65 mM using n-methylglucamine as Na+ substitute. Holding potential was −80 mV.

2.4. Statistical analysis. Pooled data are presented as mean ± SD; n denotes the number of cells. To minimize the effect of culture-to-culture variability, cells were prepared expressing each construct and data from at least three separate transfections were pooled for each comparison. An ANOVA was performed for multiple comparison, followed by a modified t test with Fisher correction (ORIGIN 10). Values of $p < 0.05$ were considered significant, in figures indicated with *.

3. Results

3.1. Patient. A 26-year-old female patient complained of hand cramps and difficulty relaxing her hands from the age of 20. She reported pain in her shoulders and soreness of forearms following exercise. There were no clear triggers and specifically neither cold nor diet exacerbated her symptoms. She had no episodic weakness. She tried mexiletine that improved her stiffness slightly but stopped it due to gastrointestinal side effects. Over the past 2 years, she had noted weakness in her hands. In terms of family history, her 54-year-old mother had cataracts removed and

129 complained of hand cramping. Neurological examination of the patient showed grip and thenar
130 percussion myotonia with warm up phenomenon and mild distal weakness. Needle EMG
131 examination revealed abundant, overlapping, myotonic discharges in most of the muscles examined
132 and myopathic motor units predominantly in her distal muscles. Short and prolonged exercise
133 testing was normal.
134 *3.2. Molecular genetic analysis.* No expansion to suggest DM1 was found in DMPK (Chr19q13.3),
135 while a pathogenic 2650 CCTG repeat diagnostic of DM2 was found in intron 1 of CNBP
136 (Chr3q21.3).
137 Analysis of the coding sequence and intron/exon junctions of CLCN1 was normal.
138 Sequencing of SCN4A revealed a missense variant of unknown clinical significance c.215C>T
139 causing a proline to leucine amino acid substitution (p.P72L) in the cytoplasmic N-terminus domain
140 (Fig. 1).
141 The c.215C>T genetic variation was not found in 200 chromosomes from 100 control individuals,
142 thus excluding the possibility of a single nucleotide polymorphism (SNP) with a frequency of more
143 than 1%. Additionally it has not been reported in the Human Genome Mutation database (HGMD;
144 http://www.hgmd.cf.ac.uk) and the NCBI's single nucleotide polymorphism (SNP;
145 http://www.ncbi.nlm.nih.gov/SNP) online database. The causative likelihood of this variant was
146 also evaluated using biophormatics program PolyPhen-2 (Polymorphism Phenotyping-2;
147 http://genetics.bwh.harvard.edu/pph2). This in silico analysis provided a PSIC score of "1"
148 (sensitivity: 0.00; specificity: 1.00), which indicates that the mutation is "probably" damaging.
149 *3.3. Functional analysis of WT and p.P72L Nav 1.4 channels.* A detailed functional characterization
150 of the SCN4A WT and mutant was performed in transiently transfected tsA201 cells in the presence
151 of the beta1 subunit. The level of the expression was highly variable, as the standard deviation
152 showed, but no differences were found in the current density measured in high sodium
153 (84.8 ± 33.6 pA/pF, $n = 19$; 124.5 ± 43.6 pA/pF, $n = 15$; 95.5 ± 48.1 pA/pF, $n = 17$ for WT, WT/P72L
154 and P72L, respectively). Fig. 2A represents typical families of current traces recorded in low

155 sodium from WT, or WT/P72L or P72L channel. We did not find any mutation-induced sustained
156 current measured 25 ms after a step to −10 mV, being the Isus 2.3 ± 1.5% of the peak current for
157 WT, 2.1 ± 1% for WT/P72L and 1.7 ± 0.5% for P72L. To characterize the kinetics of the fast
158 inactivation process, a biexponential function was used to fit the decay of the peak current traces
159 elicited by a 25 ms depolarizing pulse from −80 to −10 mV. While the fast time constant of
160 inactivation of the mutated channels was indistinguishable from the one of WT (0.7 ± 0.1 ms,
161 n = 11; 0.6 ± 0.03 ms; n = 15; 0.6 ± 0.08 ms; n = 10 for WT, WT/p72L and P72L, respectively); the
162 slow one was significantly faster (2.1 ± 0.8 ms; 0.7 ± 0.1 ms; 0.8 ± 0.1 ms for WT, WT/p72L and
163 P72L, respectively). The mutation did not affect the voltage dependence of inactivation (Vh
164 −55.71 ± 0.6 mV, n = 20; Vh −54.26 ± 0.7 mV, n = 19; Vh −54.23 ± 1 mV, n = 19 for WT, WT/P72L
165 and P72L, respectively), but in contrast caused a small hyperpolarized shift of the Vh of the voltage
166 dependence of activation (Vh −27.37 ± 2.7 mV, n = 12; Vh −31.41 ± 1.8 mV, n = 7; p = 0.005 and
167 Vh −32.60 ± 1.5 mV, n = 7, p = 7.64 10-4, for WT, WT/P72L and P72L, respectively), as shown in
168 Fig. 2B. It is well known that a shift in the voltage dependence of activation affects the overlap
169 between the activation and inactivation curve and the overlap-resulting non inactivating current, so
170 called window current, can be measured by a ramp pulse protocol. As expected, we observed a
171 consistent mutation-induced shift in the voltage dependence peak of the window current
172 (−31.7 ± 0.4 mV for WT, n = 9; −36.7 ± 0.6 for WT/P72L, n = 11 and −36.5 ± 0.4 mV for P72L,
173 n = 9) (Fig. 2C) with no change in the window current amplitude (0.1 ± 0.07 pA/pF;
174 0.08 ± 0.05 pA/pF; 0.09 ± 0.05 pA/pF for WT, WT/P72L and P72L, respectively).
175 Moreover, the mutation slowed the time course of recovery from fast inactivation (Fig. 3A). The
176 time necessary to recover the 80% of the current was 2.5 ± 0.1 ms for WT, n = 20; 4.8 ± 0.1 ms for
177 WT/P72L, n = 24 and 7.1 ± 0.2 ms for P72L, n = 23, p < 0.001. Despite the fact that in the protocol
178 we used to study of the recovery from fats inactivation, the duration of the conditioning pulse was
179 100 ms, we are aware from the literature [11] that a slower recovery from inactivation can be
180 associated with an enhanced slow inactivation process; thus we tested if this was the case of the

mutation presented here. Contrariwise, we found that the slow inactivation developed more quickly for the WT channel compared to the mutant (t1/2 being 14 ± 2 s, n = 6; 32 ± 8 s, n = 6 and 37 ± 8 s, n = 5 for WT, WT/P72L and P72L, respectively with $p < 0.05$) and the difference became permanently significant within 10 seconds of depolarization (Fig. 3B). Moreover, we tested the voltage dependence of the steady state slow inactivation (Fig. 3C) measured using a 5 second pre-pulse, followed by a 20 ms at holding potential to allow, again, the recovery from fast inactivation and a test pulse and found that the mutation induced a depolarized shift of about 4 mV (Vh −23.2 ± 3.1 mV, n = 9; Vh −27.9 ± 1 mV ($p < 0.01$), n = 7; Vh −28.7 ± 1.7 mV ($p < 0.01$), n = 7 for WT, WT/p72L and P72L, respectively). The time course of the recovery from the slow inactivation did not appear to be altered by the mutation (Fig. 3D).

Finally, considering that the mutated channels required a prolonged time to recover from the fast inactivation, we addressed the frequency-dependent modulation of the SCN4A activity by pacing the cells at 20 or at 70 Hz. While the pacing at 20 Hz did not alter the peak amplitude neither of the WT channel, nor of the mutants; when the cells were paced at 70 Hz, we found a reduction of the peak current by 20% in the WT and by 24% and 28% ($p < 0.05$) in WT/P72L and P72L channel protein (Fig. 4).

4. Discussion

In this study we report the first case of a patient positive for DM2 (2650 CCTG repeat) with a concomitant mutation in SCN4A. This novel missense mutation P72L is remarkable because it represents the first mutation described in the cytoplasmatic N terminus of Nav1.4.

This 26-year-old woman affected by DM2 presented an atypical phenotype characterized by early and severe myotonia. CLCN1 recessive mutations have been reported to enhance the myotonic phenotype in DM2 [6], [7]. However, no mutation on the CLCN1 gene was found in our patient while screening of SCN4A revealed a novel mutation c.215C>T (p.Pro72Leu) localized in the cytoplasmic N-terminus. Most mutations of Nav1.4 channel have been found in the third and fourth

homologous domain [12], whereas no mutations have been reported in the cytoplasmic N-terminus. The cytoplasmic N-terminus domain is composed of 128 amino acid residues, and it is highly conserved among all the species. Missense mutations in this domain have been described in other VGSC as in SCN1A gene (Nav1.1) [13], in SCN8A gene (Nav1.6) [14] and in SCN5A gene (Nav1.5) [15]. In SCN5A the N-cytoplasmic mutations lead to a various effect on biophysical proprieties of the channel resulting in cardiac disease as long QT syndrome or Brugada Syndrome [15].

In our case a functional alteration caused by the P72L mutation was confirmed by patch clump whole-cell recording on tsA201 cells expressing heterologous mutant channels. More specifically the most prominent effects caused by P72L mutation were a hyperpolarized shift of the voltage dependence of activation, a slower recovery of fast inactivation, a slower kinetic of entry in the slow inactivation, and a destabilized steady state of the slow inactivation.

The shift of the voltage dependence of activation increases excitability of the cell decreasing the threshold for action potential and has been reported in previous SCN4A mutations causing hyperkaliemic periodic paralysis or sodium channel myotonia [16], [17]. Since slow inactivation may have a role in determining the presence of a myotonic or a paralytic phenotype [11], [18], we investigated the development, the steady state and the recovery of slow inactivation caused by P72L substitution. Our data suggested that the mutation induced a slower entry in the slow inactivation state and that the availability curve is slightly shifted in the depolarizing direction. However compared to other mutations causing disruption of slow inactivation and paralytic episodes [19], [20], the difference is minimal. Indeed for both P72L and WT channels slow inactivation is nearly complete at 0 mV. Furthermore the mutation presented here induced a slowing in the recovery from inactivation that could compensate the “too high” availability of the channels due to the slow entry in the slow inactivated state. All these factors together, in our opinion, are in agreement with the absence of episodic weakness in our patient supporting a pure myotonic phenotype.

Although the data reported above show increased excitability we presently do not know if a lone

P72L mutation of the SCN4A gene can cause myotonia in an otherwise healthy individual because lone mutations in the N-terminal domain of Nav 1.4 have never been reported. Bearing in mind that other unknown factors may contribute to the atypical phenotype of our patient, we suggest that the mutation described may act as a modulating factor increasing the severity of myotonia.

The functional effect caused by the P72L mutation may be interpreted considering that while proline is usually solvent-exposed, despite its aliphatic chain, and it is commonly found in turns, leucine is a highly hydrophobic residue and it is usually buried within the core of the structure of a folded protein. P72 is the central residue of a triplet of proline and belongs to the region formed by the segment comprised by aminoacids 56 and 78 that is potentially disordered and unstructured (www.elm.eu.org) [21]. It has recently been recognized that many functionally important protein segments lie in regions intrinsically disordered. These short peptides can contain functional sites important for protein function that may be altered by amino acid substitution as in our case. Indeed we do not know exactly what this domain does, but our data clearly demonstrate it is involved with channel gating.

5. Conclusions

We describe the first case in the literature of associated DM2 and SCN4A mutations. The clinical practical consequence is relevant. Indeed we confirm that the presence of severe and early myotonia in DM2 should prompt investigation for other factors. If CLCN1 screening is negative this case supports for screening SCN4A gene. Further screening of SCN4A in DM2 cases with atypical myotonic phenotype is likely to reveal other cases in the future.

Authors' contribution

E. Bugiardini: drafting and revising the manuscript; study concept; interpretation of data. I. Rivolta: drafting and revising the manuscript; acquisition of data (electrophysiology), analysis of data, statistical analysis. A. Binda: acquisition and analysis of electrophysiological data. A. Soriano

Caminero: acquisition and analysis of clinical data. F. Cirillo: acquisition and analysis of data. A. Cinti: acquisition and analysis of data, construct preparation. R. Giovannoni: revising the manuscript. A. Botta: DNA analysis and interpretation of the molecular data; revising the manuscript. R. Cardani: study concept; revising the manuscript. M.P. Wicklund: study concept; acquisition of clinical data; revising the manuscript. G. Meola: study concept; revising the manuscript.

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Figure Legends

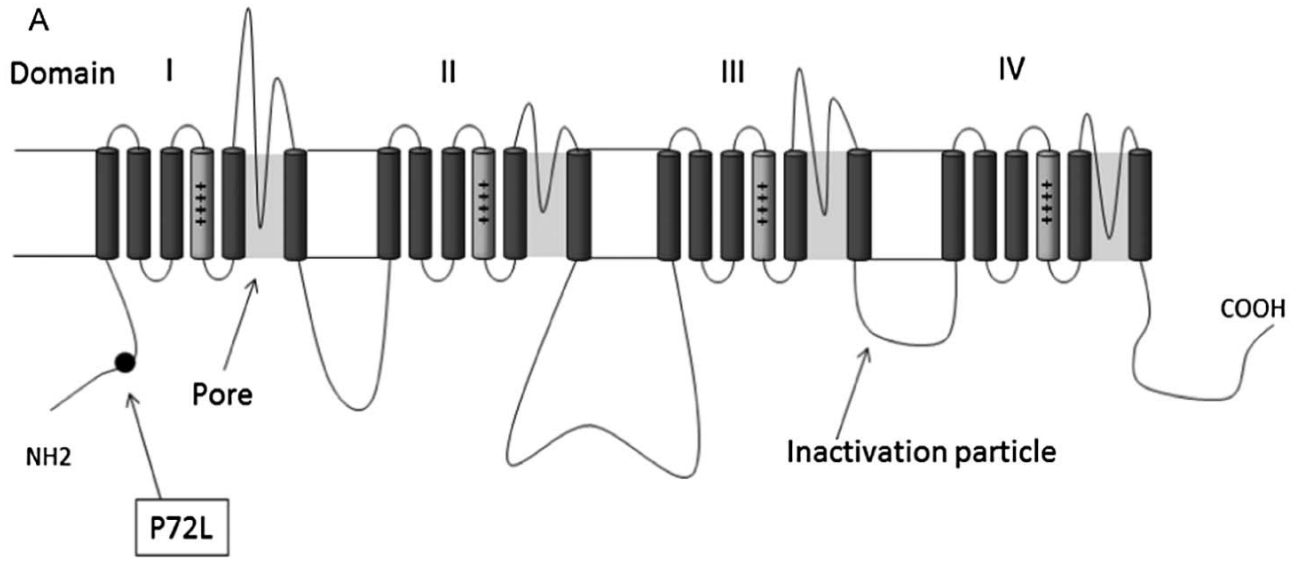
Fig. 1. Mutation p. P72L in SCN4A gene. (A) Schematic representation of the skeletal muscle sodium channel (Nav1.4) structure. P72L is located in the cytoplasmic N-terminus domain where mutations have never been described. (B) Proline 72 is well conserved amino acid among orthologs of the voltage-gated sodium channel and among several species.

Fig. 2. Biophysical characterization of wild-type and P72L mutant Nav1.4. (A) Typical families of current traces recorded in response to a series of voltage pulses from −75 mV to +10 mV, in 5-mV increment, for wild-type (WT), WT/P72L, and for P72L channels. (B) Steady state activation (filled symbols) and inactivation (empty symbols) relationships. Activation properties were determined from I/V relationship by normalizing peak INa to driving force and maximal INa, and plotting normalized conductance vs membrane voltage. Steady state activation data were fitted with a Boltzmann function. The voltage-dependence for inactivation was measured by applying a series of conditioning pulses (500 ms) from −110 mV to +20 mV in 10-mV increments before assaying current availability at a test voltage of −10 mV for 25 ms. Test voltage currents were normalized to the current measured after the −120 mV pulse and plotted versus conditioning pulse voltage. (C) Averaged sodium sensitive current was measured in response to a voltage ramp protocol (−100 to

+50 mV in 300 ms) for WT (upper trace, n = 9), WT/P72L (middle trace, n = 9) and P72L (lower trace, n = 9) channels. The dotted lines evidence the voltage at which the window current showed its maximal value.

Fig. 3. Evaluation of the recovery from fast inactivation and properties of slow inactivation. (A) Time course of recovery from fast inactivation induced by a conditioning pulse (100 ms, −10 mV) followed by a test pulse at −10 mV after a variable-duration (1–3000 ms) return to −100 mV. Plot shows amplitude of peak current, normalized to fully recovered current, as function of time after imposition of the conditioning pulse for WT (open circles, n = 20), WT/P72L (open square, n = 24) and for P72L (filled triangles, n = 23). (B) Time dependence of the onset of slow inactivation was measured using a two pulse protocol. Voltage was stepped from the holding potential of −100 mV to −10 mV for various times (P1, 500 to 50,000 ms), stepped back to −100 mV for 20 ms to let the channels recover from fast inactivation and then to −10 mV (P2, 10 ms) to record sodium current. The resulting P2/P1 ratio was plotted against the P1 duration. (C) Steady-state slow inactivation determined by a series of 5 s prepulses from −130 to +10 mV. A brief return (20 ms) to −100 mV let the channels recover from fast before the test pulse to −10 mV (WT, n = 9; WT/P72L, n = 7; P72L, n = 7). (D) Recovery from slow inactivation was determined by a 5 s depolarization pulse to −10 mV followed by a variable-duration return (50–5000 ms) to −100 mV and a test pulse to −10 mV for 10 ms to record sodium current. The data were normalized to the currents recorded with a short control pulse to −10 mV before each episode (WT, n = 9; WT/P72L, n = 12; P72L, n = 10).

Fig. 4. Response of the WT, WT/P72L and P72L channels to pulse train stimulation. (A and B) Peak currents were recorded applying a use-dependent protocol pulsing at −10 mV, at 20 Hz and 70 Hz respectively and normalizing each peak amplitude to the 1st step amplitude. For WT (open circle) n = 14; for WT/P72L (open squares) n = 18 and for P72L (filled triangles) n = 20. (C) Typical first and last (200th) steps evoked by a 70 Hz use-dependent protocol.



B

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Nav1.4:                DLEAGKNLPMIYGDPPPEVIGIPLEDL
Nav1.5:                DLQASKKLPDLYGNPPQELIGEPLEDL
Nav1.6:                DLEAGKSLPFIYGDIPQGLVAVPLEDF
Nav1.1:                DLEAGKNLPFIYGDIPPEMVSEPLEDL
Nav1.2:                DLEAGKSLPFIYGDIPPEMVSVPLEDL
Nav1.3:                DLEAGKNLPFIYGDIPPEMVSEPLEDL
Nav1.7:                DLEAGKQLPFIYGDIPPGMVSEPLEDL
Nav1.8:                DLKACNQLPKFYGEIPAELIGEPLEDL
Nav1.9:                DLKASRKLPKLYGDIPRELIGKPLEDL
Rattus norvegicus:     DLEAGKNLPLIYGDPPPEVIGIPLEDL
Mus musculus:          DLEAGKNLPLIYGDPPPEVIGVPLEDL
Thamnophis sirtalis:   DLEQGKGLPLIYGEPPPELIGVPLEDL
Tetraodon nigroviridis: DLEAGKSLPFIYGDIPPHLVSIPLEDI
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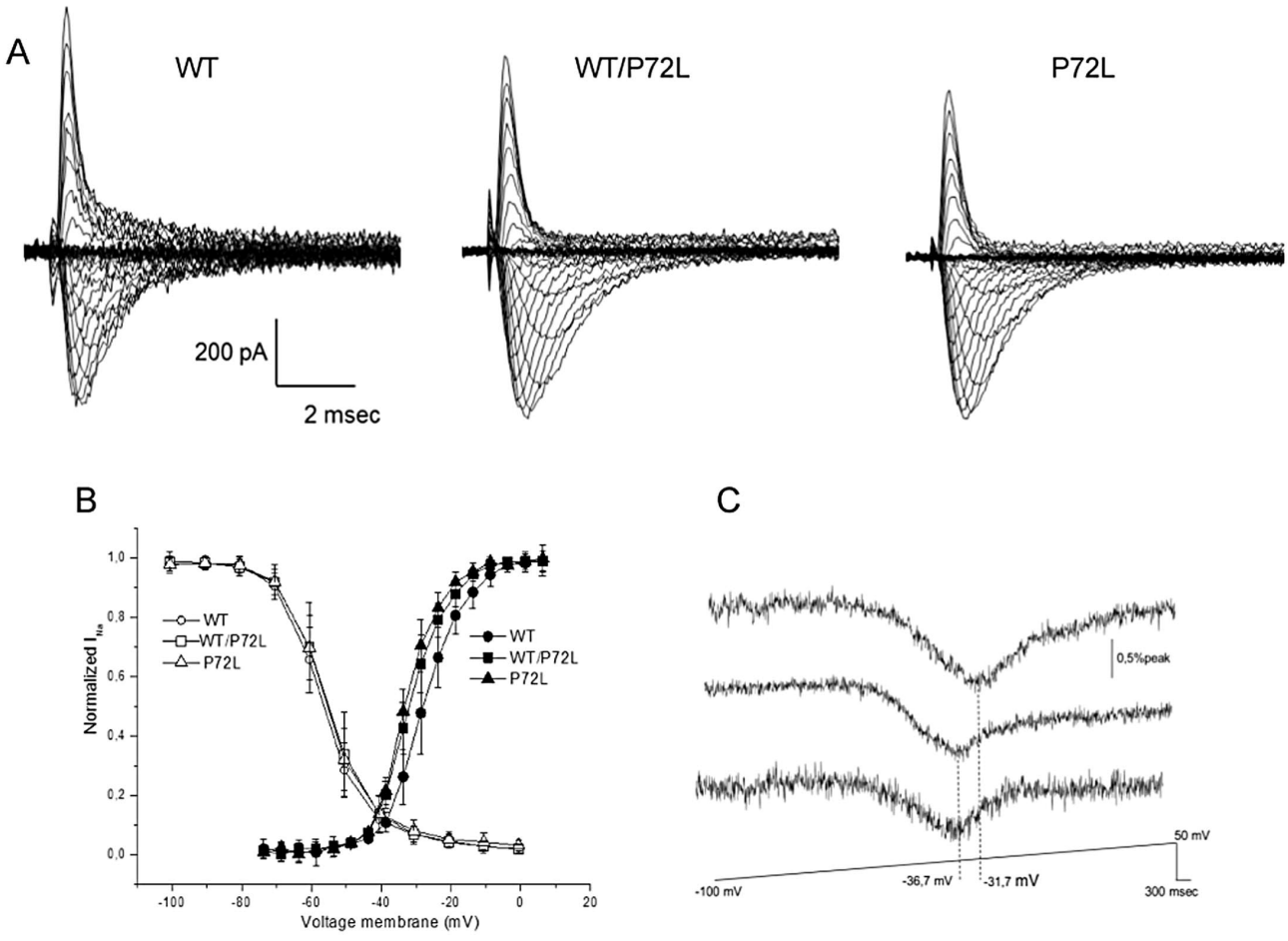
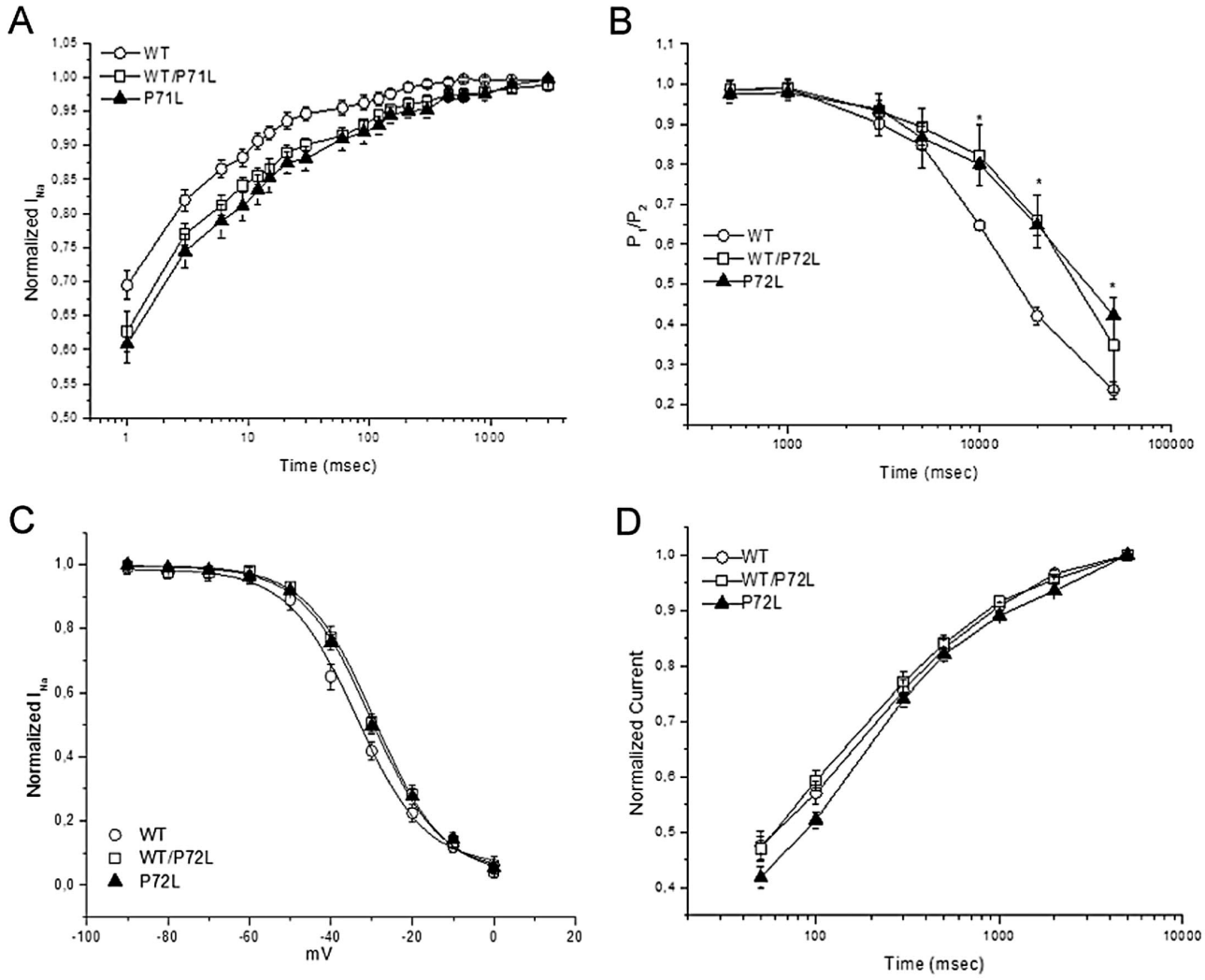
362

363 Figure 1



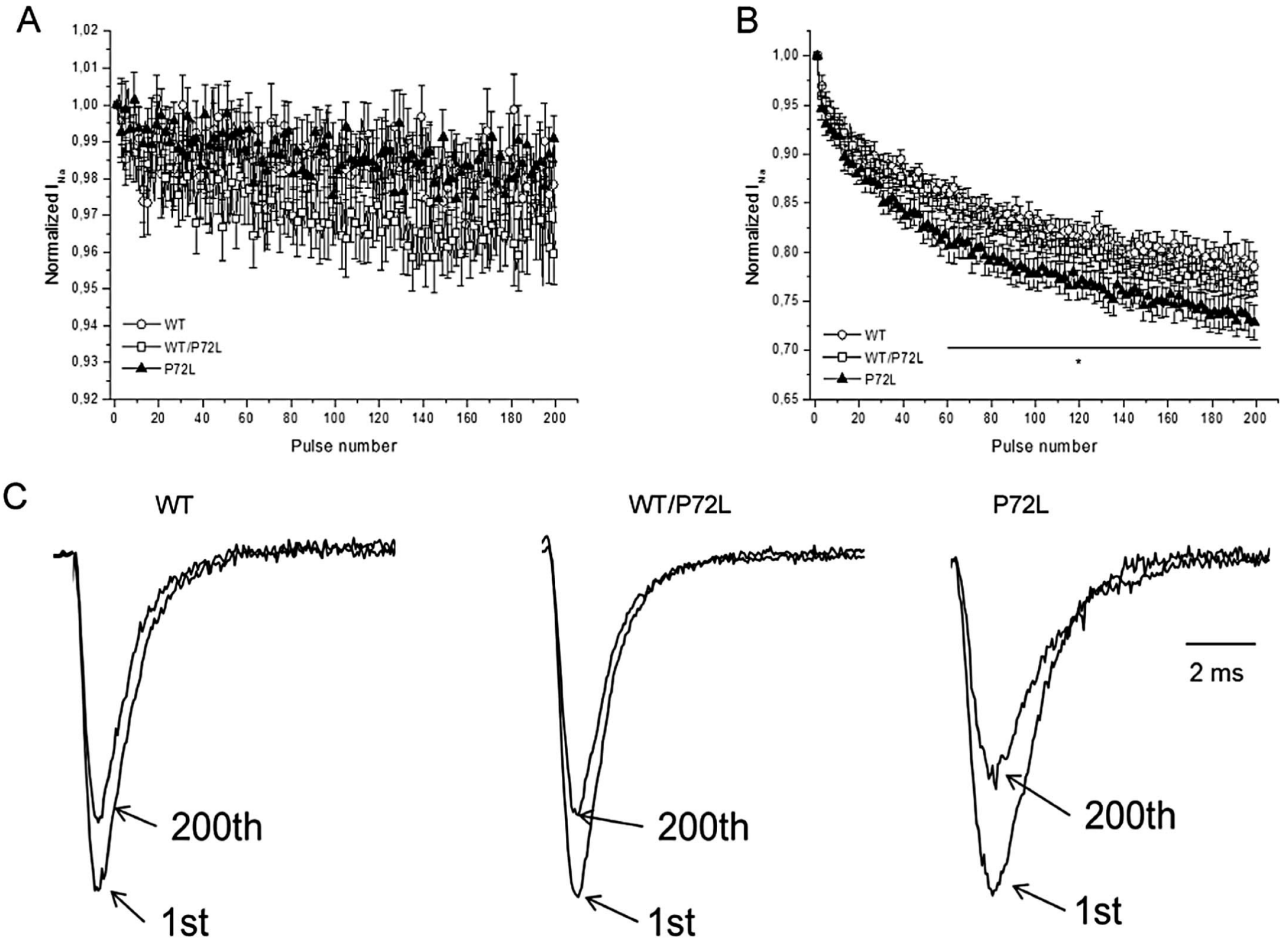
364

365 Figure 2



366

367 Figure 3



368

369 Figure 4