Translational approach to address therapy in myotonia permanens due to a new SCN4A mutation

ABSTRACT

Objective: We performed a clinical, functional, and pharmacologic characterization of the novel p.P1158L Nav1.4 mutation identified in a young girl presenting a severe myotonic phenotype.

Methods: Wild-type hNav1.4 channel and P1158L mutant were expressed in tsA201 cells for functional and pharmacologic studies using patch-clamp.

Results: The patient shows pronounced myotonia, slowness of movements, and generalized muscle hypertrophy. Because of general discomfort with mexiletine, she was given flecainide with satisfactory response. In vitro, mutant channels show a slower current decay and a rightward shift of the voltage dependence of fast inactivation. The voltage dependence of activation and slow inactivation were not altered. Mutant channels were less sensitive to mexiletine, whereas sensitivity to flecainide was not altered. The reduced inhibition of mutant channels by mexiletine was also observed using clinically relevant drug concentrations in a myotonic-like condition.

Conclusions: Clinical phenotype and functional alterations of P1158L support the diagnosis of myotonia permanens. Impairment of fast inactivation is consistent with the possible role of the channel domain III S4-S5 loop in the inactivation gate docking site. The reduced sensitivity of P1158L to mexiletine may have contributed to the unsatisfactory response of the patient. The success of flecainide therapy underscores the usefulness of in vitro functional studies to help in the choice of the best drug for each individual.

GLOSSARY

DM1 = myotonic dystrophy type 1; IC50 = half-maximum inhibitory concentration; TB = tonic block; UDB = use-dependent block; UDR = use-dependent reduction; WT = wild-type.

Gain-of-function missense mutations of the skeletal muscle Nav1.4 sodium channel cause myotonia or flaccid weakness. Impaired fast-inactivation of hNav1.4 mutants likely induces myotonia, while enhancement of activation and impaired slow inactivation contribute to paralytic attacks. Various substitutions at the same amino acid (Gly1306) induce various degrees of channel alteration, which correlate to the severity of symptoms ranging from the mild myotonia fluctuans (G1306A) to the severe myotonia permanens (G1306E). Unusual phenotype is also explained by specific behavior of channel mutant. Carriers of the P1158S mutation show myotonia in a warm environment and paralytic attacks at cold temperatures. Accordingly, heterologously expressed P1158S channels show alteration of activation and slow inactivation at 22°C but not 37°C.

The sodium channel blocker mexiletine is the preferred drug to alleviate myotonia. Nevertheless, in case of side effects or lack of efficacy, mexiletine can be substituted for by another sodium channel blocker. We previously demonstrated that flecainide is successful in mexiletine-low responsive patients carrying G1306E, likely due to mutation-specific gating changes.

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We report the clinical, functional, and pharmacologic characterization of a new hNav1.4 mutation, p.P1158L, found in a severely affected girl with symptoms resembling myotonia permanens. Gating alteration of heterologously expressed P1158L channels is consistent with the phenotype. The patient remained unsatisfied with mexiletine, but claimed great improvement with flecainide. Accordingly, P1158L channels are less sensitive to mexiletine in vitro but show unaltered flecainide sensitivity. These results highlight how in vitro pharmacologic study of hNav1.4 mutants can help in better addressing treatment in myotonic patients.

METHODS Genetic analysis. After written informed consent, genomic DNA was extracted from peripheral blood. DMI genetic analysis was performed following the standard diagnostic method. All the coding exons and intron–exon junctions of SCN4A and CLCN1 were amplified (primer sequences and conditions are available upon request). The PCR fragments were directly sequenced using Big Dye Terminator Cycle Sequencing Kit in an automated sequencer 3130 (Applied Biosystems, Foster City, CA). SeqScape software (Applied Biosystems) was used to align and compare the sequences with National Center for Biotechnology Information control sequences (NG_011699 and NG_0095815 and NG_008083 for SCN4A, CLCN1). To confirm the result obtained on exon 19 of SCN4A, amplification and sequencing were repeated and the variant was checked in the father.

Mutagenesis and expression of recombinant sodium channels. The pRe/CMV-hNav1.4 vector encoding the wild-type (WT) skeletal muscle voltage-gated sodium channel was provided by Desaphy et al. The P1158L mutant was engineered using the QuickChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) and was confirmed by complete sequencing. The WT or P1158L hNav1.4 plasmids (1 μg/100-mm dish) were transiently coexpressed in tsA201 cells with the pCD8-IRESH-B1 plasmid containing the CD8 receptor and the auxiliary sodium channel β1 subunit (0.5 μg/100-mm dish), using the calcium phosphate method. Microbeads coated with anti-CD8 antibody (Dynal, Invitrogen, Milan, Italy) were used to identify cells for patch clamp experiments.

Whole-cell recording and data analysis. Sodium currents (I\textsubscript{Na}) were recorded in whole-cell patch-clamp configuration at room temperature (20°C–22°C), as previously described. The pipette solution contained (in mM) 120 CsF, 10 CsCl, 10 NaCl, 5 EGTA, and 5 Cs-HEPES (pH 7.2). The bath solution contained (in mM) 150 NaCl, 4 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 5 Na-HEPES, and 5 glucose (pH 7.4). Patch pipettes had resistance ranging from 1 to 3 MΩ. Capacitance currents were partially compensated using the amplifier circuit. Only those data obtained from cells exhibiting series resistance errors <5 mV were considered for analysis.

Mexiletine-HCl and flecainide-acetate salt (Sigma-Aldrich, Milan, Italy) were solubilized in bath solution at the final concentration. The patched cell was exposed to a continuous stream of control or drug-supplemented bath solution. A maximum of 2 drug concentrations were tested on each cell, to minimize the possible bias due to I\textsubscript{Na} rundown, as previously described. Because of the known spontaneous shift of voltage dependence during whole-cell experiments, much care was taken to perform the various protocols respecting a constant sequence to allow comparison between the cells.

Data analysis was performed using pCLAMP 10.3 (Axon Instruments, Union City, CA) and SigmaPlot 8.02 (Systat Software GmbH, Erkzeh, Germany). The I\textsubscript{Na} decay was fit to a single exponential function, an exponential function, or a Boltzmann factor. Because slow inactivation is not complete, a nonzero residual current (I\textsubscript{b}) was introduced in the function 

\[ G/I_{Na,x}= \frac{I_b+(1-I_b)exp(-(V-V_50)/S)} {1+exp((V-V_50)/S)} \]

(Boltzmann function, equation 3), where \( V_50 \) is the midpoint and \( S \) is the slope factor. The complete voltage dependence relationships were obtained in each cell, and the fit parameters were averaged as mean ± SEM from n cells. The relationships were fit to a first-order binding function

\[ \frac{IC_{50}}{IC_{50}^{drug}} = \frac{1}{1+(\text{drug}/IC_{50})^{n}} \]

(equation 4), where IC\textsubscript{50} is the half-maximum inhibitory concentration and \( n \) is the logistic slope factor. The fit parameter values are reported together with the SE of the regression.

RESULTS Case report. The proband was born in Algeria of consanguineous parents (the paternal grandmother and the maternal great-grandmother were first-line cousins) coming from a village of nearly 3,000 inhabitants (figure e-1 on the Neurology® Web site at Neurology.org). She did not require resuscitation at birth, but her mother noticed feeding difficulties in the neonatal period, facial grimaces during crying, and convergent strabismus. When she was 9 years of age, an Algerian pediatrician reported generalized muscle hypertrophy with facial dysmorphism, including narrow palpebral fissures and small mouth. Short and hypertrophic neck, slender hands, flat feet, and kyphoscoliosis were described together with statural and ponderal growth delay. CK level was elevated (about 600 UI/L). EMG showed subcontinuous myotonic discharges. Echocardiogram was unremarkable. Because slow inactivation is not complete, a nonzero residual current (I\textsubscript{b}) was introduced in the function

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and elbow retraction. Finger flexors myotonic stiffness hindered hands opening. Voluntary and percussion myotonia at hands lasted more than 5 seconds. Eyelid myotonia was striking with marked lid lag and convergent strabismus. Extrinsics eye muscles myotonia did not allow her to follow the examiner’s finger by eyes. Tongue myotonia was so pronounced that she could not oscillate the protruded tongue and swallowing was slow. No muscle weakness was observed or reported, except for orbicularis oculi. Muscle stiffness was severe and exacerbated by cold temperatures, limiting all daily activities. EMG showed continuous repetitive myotonic discharges in absence of myopathic changes. No significant compound muscle action potential amplitude changes were observed upon 3-Hz repetitive nerve stimulation.12

Family history was remarkable for the proband father, who showed muscle stiffness, facial muscle weakness, including orbicularis oculi and oris, mild symmetrical palpebral ptosis, distal and semidistal limb muscles weakness (3.5 score on the Medical Research Council scale), and muscle hypotrophy. There was neither facial muscle myotonia nor lid lag phenomenon. Voluntary and percussion myotonia at hands lasted for more than 5 seconds. EMG revealed myopathic changes and myotonic runs. These data were compatible with the diagnosis of myotonic dystrophy type 1 (DM1), which was confirmed by genetic analysis with the presence of 250 CTG repeats at the DMPK locus. The proband was analyzed for both DM1 and SCN4A mutations. The genetic analysis showed the presence of 310–390 CTG repeats and a new SCN4A mutation in exon 19 (c.3473C>T, p.Pro1158Leu), indicating the coexistence of DM1 and sodium channel myotonia. The SCN4A mutation was absent in the father, while the mother was not available for genetic analysis. Nevertheless, no clinical sign of myotonia was found in the mother, thereby suggesting that p.Pro1158L was a de novo mutation in the proband. The p.Pro1158Leu was not described in public databases (i.e., ExAC and 1000 G) and was absent in 140 alleles from North African controls and in 350 alleles from Italian controls. Bioinformatic analysis using Polyphen2 and Mutation Taster prediction software classified the variant as probably damaging.

To allow comparison of channel pharmacology to previous data,3 we measured inhibition of \( I_{Na} \) elicited at \(-30\) mV from an holding potential of \(-120\) mV, every 10 (0.1 Hz) or 0.1 (10 Hz) seconds. Mexiletine (300 \( \mu M \)) produced a minor inhibition of P1158L \( I_{Na} \) at 0.1 Hz (\(-56.1\%\), \( n = 6, p < 0.001 \)) and 10 Hz (\(-16.0\%\), \( n = 6, p < 0.01 \)) compared to WT (figure 3, A–C). The concentration-response relationships were shifted toward greater mexiletine concentrations for P1158L channels (figure 3D). At 0.1 Hz, the half-maximum inhibitory concentration (\( IC_{50} \)) of mexiletine was 283 ± 60% inhibition of \( I_{Na} \) at 0.1 Hz and \(-80\%\) inhibition at 10 Hz (figure 3, E–G). Mexiletine effects
on P1158L channels were not statistically different from those exerted on WT. At 0.1 Hz, the IC50 was 76 ± 7 μM for WT and 63 ± 2 μM for P1158L; at 10 Hz, it was 34 ± 4 μM for WT and 28 ± 1 μM for P1158L (figure 3H).

We also tested the drugs in a condition more similar to a myotonic discharge of action potentials: the holding potential was −90 mV, the test pulse lasted 5 ms and was applied at 50 Hz frequency. In absence of drug, such a protocol induced a use-dependent reduction (UDR) of INa amplitude by 38 ± 3% (n = 34, not shown). The drugs were applied to INa first during 0.1 Hz stimulation to develop tonic block (TB), then during 200 test pulses at 50 Hz to measure the use-dependent block (UDB). The net effect of drug was calculated offline by subtracting UDR from UDB and further adding TB, as previously detailed. The time course of net drug effect on WT and P1158L INa is shown in figure 4, A and B. The data point at time zero represents TB, which increased with drug concentration. During the following sweeps at 50 Hz, the INa amplitude was significantly longer for P1158L compared to WT (at least p < 0.05 with unpaired Student t test).
decreased to a steady state. Inhibitory effect of 10 and 30 \( \mu \)M mexiletine was significantly less pronounced on P1158L channels compared to WT, resulting in a left shift of the concentration-response curve for P1158L (figure 4, A and C). In contrast, little difference was found between the 2 channels for flecainide (figure 4, B and C).

**DISCUSSION** Clinically, the proband’s phenotype was comparable to myotonia permanens, which is characterized by severe myotonia aggravated by cold, onset near birth with possible breathing difficulties, and subcontinuous myotonic EMG.15 Two other SCN4A mutations have been linked to myotonia permanens: G1306E and Q1633E.15,16 Patients with more severe cases may encounter life-threatening severe neonatal episodic laryngospasm.16–19 Conversely to her father, the proband did not show clinical manifestation related to myotonic dystrophy except for the orbicularis oculi weakness. Neither weakness nor EMG myopathic changes were detected in other muscles. Both mexiletine and flecainide were ineffective in changing this clinical sign. Yet we cannot exclude that the DM1 and SCN4A mutations may compound to increase the severity of her myotonia, as recently suggested.20,21

The P1158L mutation found in the proband is located in the intracellular linker between the fourth and fifth segment of domain III of hNav1.4. Another mutation at this position, P1158S, is associated with myotonia in warm environment and paralytic attacks in cold conditions.3 The P1158S channel shows normal behavior at 37°C and alteration of activation and slow inactivation at 22°C.4,5 Other mutations close to Pro1158 include A1152D associated with paramyotonia congenita22 and I1160V linked to sodium channel myotonia23,24 or paramyotonia congenita (Lo Monaco, unpublished data). Those mutations affect the fast inactivation and deactivation of the channel. Indeed, the short S4-S5 loop of domain III is thought to form the docking site of the DIII-DIV loop acting as the inactivation gate.25 Accordingly, we found that P1158L induced a slower rate of current decay and a depolarized shift in the voltage dependence of availability, which are common defects associated with sodium channel myotonia, while the lack of effect on activation and slow inactivation is consistent with the lack of paralytic episodes in the patient.1 Although proline and leucine are both hydrophobic residues, proline cannot form hydrogen bonding with its embedded backbone nitrogen.

### Table

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<td>( V_{50} )</td>
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<td>( V_{50} )</td>
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Abbreviations: \( I_0 \) = residual current; S = slope factor; \( V_{50} \) = half-maximum voltage. Values are mean ± SEM. The \( p \) value was calculated using unpaired Student t test.
Figure 3  Effects of mexiletine and flecainide on wild-type (WT) and P1158L hNav1.4 channels

(A, B) Representative WT and P1158L current traces recorded at steady-state before and during application of 300 μM mexiletine at 0.1 and 10 Hz stimulation frequencies. (C) Box and whisker chart representation of mean effects of 300 μM mexiletine at both stimulation frequencies. Using unpaired Student t test, inhibition of P1158L channels was significantly lower compared to WT. (D) The concentration-effect relationships for mexiletine on WT and P1158L, fitted to a first-order binding function (equation 4 in Methods), confirmed the lower sensitivity of P1158L channels to mexiletine. (E, F) Representative WT and P1158L current traces recorded at steady-state before and during application of 100 μM flecainide at 0.1 Hz stimulation frequencies. (G) Box and whisker chart representation of mean effects of 100 μM flecainide at both stimulation frequencies. No significant difference was found between WT and P1158L channels. (H) The concentration-effect relationships for flecainide on WT and P1158L, fitted to a first-order binding function, were superimposed.
Thus the substitution by leucine likely modifies the architecture of the S4-S5 loop. The introduction of the hydrophilic serine may determine a different alteration of the loop, which may explain the different effects of P1158L and P1158S on channel behavior and consequently on clinical phenotype. Importantly, P1158L produced channel defects similar to those of the 2 other myotonia permanens...
mutations. The rightward shift of the midpoint of fast inactivation voltage dependence was 9, 10.7, and 12.6 mV for P1158L, Q1633E, and G1306E, respectively. In addition, the current decay was greatly slowed by all 3 mutations.

Although the proband showed a partial response to mexiletine upon clinical examination, she complained of general discomfort and asked for treatment interruption. We and others have previously demonstrated that myotonic mutations can affect the effect of mexiletine, either due to altered intrinsic affinity or to mutation-induced altered gating. By inducing a leftward shift of channel availability voltage dependence, the myotonia permanens G1306E mutation is less sensitive to mexiletine compared to WT channels. We further demonstrated that, instead, the mutation did not affect the sensitivity to flecainide, and this eventually allowed a successful shift of therapy from mexiletine to flecainide in a mother and her son, both carrying G1306E and showing a very limited response to mexiletine. We also recently demonstrated in a pharmacologically induced rat model of myotonia that flecainide is an efficient antimonytoxic drug at clinically relevant doses. On the basis of this experience, the P1158L carrier was given flecainide. She remained very satisfied, claiming an attenuation of stiffness incomparably greater and more stable than with mexiletine.

In vitro, P1158L channels showed a reduced affinity to mexiletine compared to WT. As for G1306E, we can hypothesize that the reduced affinity is, at least in part, due to the rightward shift of the voltage dependence of fast inactivation: the minor proportion of inactivated P1158L channels at the holding potential, compared to WT, may reduce the apparent affinity of mutant channels to mexiletine. Blockade of P1158L channels by mexiletine is also reduced in a myotonic-like condition at the clinically relevant drug concentration of 10 μM. The human therapeutic blood concentration range is 4–11 μM for mexiletine and 0.5–2 μM for flecainide. In contrast, the mutation did not significantly alter the block exerted by flecainide.

In this study, we demonstrate that the new P1158L hNav1.4 mutation is likely responsible for myotonia. Both the clinical phenotype and the biophysical channel defects are consistent with myotonia permanens. The reduced apparent affinity of the mutant channel to mexiletine may have contributed to the unsatisfactory response of the patient to the drug. Together with the previous experience with G1306E carriers, this result suggests that most of the mutations showing a rightward shift of fast inactivation voltage dependence may greatly benefit from flecainide treatment. The success of therapy with flecainide further supports the development of precision medicine for the treatment of myotonic syndromes, in which the functional study of the mutant protein at bench may be helpful to choose the best drug for each individual.

**AUTHOR CONTRIBUTIONS**

J.-F. Desaphy: study concept and design, data and statistical analysis, writing the manuscript. R. Carbonara: patch-clamp data collection and analysis. A. Modoni: clinical examination of proband and familiar. J. Roussel: patch-clamp data collection and analysis. P. Imbrici: patch-clamp data collection and analysis. A. D’Amico: clinical examination of proband and familiar. S. Pagliarani: genetic analysis. S. Lucchiari: genetic analysis. M. Lo Monaco: clinical examination of proband and familiar; study concept and design, writing the manuscript. D. Conte Camerino: study concept and design; revising the manuscript for intellectual content.

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**DISCLOSURE**

The authors report no disclosures relevant to the manuscript. Go to Neurology.org for full disclosures.

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**REFERENCES**


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