Mice with an Na\(_{\text{V}1.4}\) sodium channel null allele have latent myasthenia, without susceptibility to periodic paralysis

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Over 60 mutations of SCN4A encoding the Na\(_{\text{V}1.4}\) sodium channel of skeletal muscle have been identified in patients with myotonia, periodic paralysis, myasthenia, or congenital myopathy. Most mutations are missense with gain-of-function defects that cause susceptibility to myotonia or periodic paralysis. Loss-of-function from enhanced inactivation or null alleles is rare and has been associated with myasthenia and congenital myopathy, while a mix of loss and gain of function changes has an uncertain relation to hypokalaemic periodic paralysis. To better define the functional consequences for a loss-of-function, we generated Na\(_{\text{V}1.4}\) null mice by deletion of exon 12. Heterozygous null mice have latent myasthenia and a right shift of the force-stimulus relation, without evidence of periodic paralysis. Sodium current density was half that of wild-type muscle and no compensation by retained expression of the foetal Na\(_{\text{V}1.5}\) isoform was detected. Mice null for Na\(_{\text{V}1.4}\) did not survive beyond the second postnatal day. This mouse model shows remarkable preservation of muscle function and viability for haploinsufficiency of Na\(_{\text{V}1.4}\), as has been reported in humans, with a propensity for pseudo-myasthenia caused by a marginal Na\(^+\) current density to support sustained high-frequency action potentials in muscle.

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Abbreviations: CMAP = compound muscle action potential; HypoKPP = hypokalaemic periodic paralysis

Introduction

The voltage-activated sodium channel of skeletal muscle, Na\(_{\text{V}1.4}\), is essential for the generation of propagated action potentials that initiate Ca\(^{2+}\) release and contraction throughout the length of the fibre. Over 60 mutations of the SCN4A gene encoding Na\(_{\text{V}1.4}\) have been associated with several clinically distinct channelopathies of skeletal muscle (Lehmann-Horn et al., 2004; Horga et al., 2013; Cannon, 2015). Most mutations are missense substitutions that produce gain-of-function changes by altering channel gating that governs the transitions between closed, open, and inactivated states. The most frequent defect is a partial disruption of inactivation (Cannon et al., 1991; Yang et al.,...
1994), in other cases activation is enhanced (Cummins et al., 1993), and either defect will cause an excess of inward Na⁺ current that depolarizes the fibre. When the magnitude of these gain-of-function changes is mild, fibres are hyperexcitable with a tendency to repetitively discharge in long bursts of many seconds in response to a single stimulus. These trains of pathologic after-discharges produce myotonia, an involuntary sustained after-contraction. More severe gain-of-function defects cause instability of the resting potential with susceptibility to attacks of periodic paralysis (Cannon et al., 1993). The episodes of weakness are often in response to environmental trigger factors (exercise, shift of extracellular K⁺, cooling, or stress) that elicit a pathological sustained depolarization to about −50 mV, which renders the fibre inexcitable and refractory from firing action potentials because of Na⁺ channel inactivation. The pathogenic nature of these gain-of-function changes has been confirmed by the creation of a mouse model with a targeted mutation of NaV1.4 (M1592V) that has hyperkalaemic periodic paralysis (HyperKPP) with myotonia (Hayward et al., 2008), and by computer simulation demonstrating the gating changes are sufficient to produce myotonic discharges or depolarization-induced loss of excitability in model fibres (Cannon et al., 1993).

Loss-of-function changes for disease-associated mutations of NaV1.4 are less common, and the consequences of these defects on fibre excitability are not firmly established. The first reports in which loss-of-function was the primary defect were from expression studies of missense mutations at arginine residues in the S4 voltage sensor of domain II in patients with hypokalaemic periodic paralysis (HypoKPP) (Jurkat-Rott et al., 2000; Struyk et al., 2000). Inactivation was enhanced by a modest hyperpolarized voltage shift of ~10 mV and led to the proposal that NaV1.4 loss-of-function defects caused susceptibility to HypoKPP whereas gain of function changes caused myotonia and HyperKPP. Later, the HypoKPP mutant channels were discovered to also be leaky with an anomalous conduction pathway in the gating pore (Sokolov et al., 2007; Struyk and Cannon, 2007). The leak is a variant for a gain-of-function, and so the interpretation of a possible pathogenic role for the loss-of-function changes in the conventional Na⁺-conducting pore was less certain. A more severe loss-of-function produced by a ~30 mV shift and a 10-fold slower recovery from inactivation was then discovered in three unrelated probands with congenital myasthenia (two cases being recessive) with ptosis, fatigable weakness, and abnormal motor responses to repetitive nerve stimulation (Tsujiro et al., 2003; Arnold et al., 2015; Habbout et al., 2016). The anomalously enhanced inactivation produces near absence of function for the mutant allele. Finally, whole exome sequencing in a cohort of patients with infantile hypotonia and congenital myopathy revealed a recessive inheritance pattern of Nav1.4 homozygous and compound heterozygous null alleles or loss-of-function mutants (Zaharieva et al., 2015). This broad spectrum of clinical phenotypes associated with loss-of-function mutations for NaV1.4 raises questions about pathomechanism and genotype-phenotype associations. To address these questions, we generated mice with a deletion of exon 12 in SCN4A. Heterozygous (+/Δ_Ex12) mice are viable, but have reduced Na⁺ current density and latent myasthenic features without susceptibility to periodic paralysis. Homozygous NaV1.4 null mice do not survive beyond postnatal Day 2 and none of the patients with two null alleles survived beyond a few hours. These results suggest that an NaV1.4 partial loss-of-function may cause myasthenic weakness and that viability is preserved even with haploinsufficiency, but a homozygous null is neonatal lethal.

**Materials and methods**

**Generation of Δ_Ex12 mice**

The NaV1.4-R669H mouse we previously created as a knock-in mutation by homologous recombination (Wu et al., 2011) also contained LoxP sites that flanked exon 12. In that prior study, numbering started with exon 1 contained entirely in the 5’ untranslated region (transcript ID, ENSMUST00000106818), and hence the floxed exon was previously identified as number 13. Subsequently, we have used the revised convention of designating exon 1 as the one containing the initiating methionine in the coding region (Transcript ID, ENSMUST0000021056). The 5’-loxp site was 52 bp upstream from exon 12 and the 3’-loxp site was 202 bp downstream from exon 12. Heterozygous (+/NaV1.4-R669H) mice were crossed with Meox2 tm1(cre)/Sor mice (Tallquist and Soriano, 2000) that express Cre recombinase on embryonic Day 5 to generate mice with the NaV1.4 null allele (+/Δ_Ex12). The heterozygous mice (+/Δ_Ex12) were back-crossed with 129/Sv mice for more than 10 generations to produce a congenic line. Mice aged 2–8 months were used for the physiological studies.

Genotyping was performed on tail-snip DNA using PCR amplification with intronic primers flanking exon 12 (forward, 5’- GCCCTTCGTCCCAAAAAGCTGCTGGAAG -3’; reverse, 5’- CCTAAGCCTGGTCTACTAATCTC -3’). PCR products were resolved on a 1% agarose gel and the mutant Δ_Ex12 allele was visualized as the smaller band (212 bp) compared to wild-type (525 bp).

**Sodium current recordings in dissociated fibres**

Sodium currents were recorded from fibres of the flexor digitorum brevis using a two-electrode voltage clamp (Axoclamp 2B) as previously described (Fu et al., 2011). Briefly, the flexor digitorum brevis was rapidly dissected and dissociated in Dulbecco’s modified Eagle medium (DMEM) plus collagenase (1 mg/ml) supplemented with 1.8 mM Ca²⁺, followed by titration with a polished pipette. Fibres were maintained in DMEM plus 10% foetal bovine serum, 1% glutamine, 100 U/ml penicillin-streptomycin and 10 mM HEPES at 37°C in 5% CO₂ and voltage-clamp recordings were performed the following day. Just prior to recording, the fibre transverse tubule system was disrupted by exposure to DMEM plus...
400 mM glycerol for 1 h to improve the voltage-clamp control of membrane potential. Currents were recorded in a Cl\(^-\)/C\(_0\)-free low Na\(^+\) bath to minimize series resistance errors; 10 mM NaOH, 130 mM TEAOH, 1 mM Mg(OH)\(_2\), 1 mM Ca(OH)\(_2\), 10 mM HEPES, 10 mM glucose, 5 mM 4-aminopyridine (4-AP), 0.1 mM anthracene-9-carboxylate, and 5 \(\mu\)M nifedipine, pH adjusted to 7.4 with methanesulphonic acid. Subtraction of background currents was performed by measuring the residual currents after a depolarized conditioning pulse to inactivate Na\(^+\) channels, as previously described (Fu et al., 2011).

**Muscle excitability measured by the compound muscle action potential**

The compound muscle action potential (CMAP) was measured in two different preparations. The CMAP recorded in response to repetitive nerve stimulation as a test of the fidelity of neuromuscular transmission was performed with an *in vitro* isolated muscle preparation (Fig. 5). The soleus muscle with an intact distal motor nerve was mounted in a tissue bath of the same composition as in the contraction studies, except D-tubocurarine was either omitted or applied at a concentration of 0.5 \(\mu\)M, and the nerve was stimulated with a suction electrode. The CMAP was measured with a monopolar EMG electrode inserted into the muscle and AC coupled to a differential amplifier (Grass Instruments P511). The nerve was stimulated with 1 ms current pulses applied at frequencies of 2–100 Hz. In the second preparation, the CMAP was measured *in vivo* from muscles of the posterior leg (gastrocnemius and soleus) in response to sciatic nerve stimulation. The animal was maintained under isoflurane anaesthesia during the procedure, as previously described (Wu et al., 2011), and a glucose plus insulin challenge was administered intravenously.

**Study approval**

All procedures were in accordance with animal protocols approved by the Institutional Animal Care and Use Committees at the University of Texas Southwestern Medical Center and the David Geffen School of Medicine at UCLA.

**Results**

**Generation of exon 12 deleted mice**

A null allele for the skeletal muscle sodium channel was created by deletion of exon 12 in *SCN4A* that encodes the pore-forming \(\alpha\)-subunit of Na\(_V\)1.4 (Fig. 1). We previously generated a knock-in missense mutation in exon 12 by homologous recombination (Wu et al., 2011) to produce a mouse model for HypoKPP (Na\(_V\)1.4-R669H). The targeting construct also contained LoxP sites flanked exon 12. Heterozygous (+/NaV1.4-Ex12) mice were viable, fed and gained weight normally, had no gross motor deficits, and bred successfully. Crosses of +/NaV1.4-Ex12 and wild-type mice yielded heterozygous (+/Δ_Ex12) animals at the expected ratio.
Mendelian frequency of ~50%. A sample of 140 pups at postnatal Days 0–1 from (+/Δ_Ex12) × (+/Δ_Ex12) yielded 118 live mice: 34% (+/+, wild-type), 59% (+/Δ_Ex12), 7.6% (Δ_Ex12/Δ_Ex12). Of the 22 expired mice, 68% were homozygous for Δ_Ex12. Homozygous Δ_Ex12 mice never survived beyond postnatal Day 2. None of the neonatal mice had kyphosis, wrist drop, or small body size that commonly results from decreased foetal movements (Pai, 1965; Brandon et al., 2003).

Sodium currents are reduced in heterozygous +/Δ_Ex12 mice

Exon 12 contains 174 bp of coding sequence for transmembrane segments spanning the carboxyl half of S4 in the second homologous domain (DII) of the channel. While deletion of exon 12 is predicted to preserve the reading frame, the mutant transcript is expected to be non-functional as most of the DII voltage sensor is missing. Sodium currents were measured with a two-electrode voltage clamp in single fibres dissociated from the flexor digitorum brevis muscle of wild-type or +/Δ_Ex12 mice. Peak Na⁺ current was divided by cell capacitance to compensate for variation in fibre size. This normalized Na⁺ current is plotted as a function of the test depolarization from a holding potential of −100 mV (Fig. 2A). Sodium current recorded from a +/Δ_Ex12 fibre in response to a test depolarization from −120 mV to −40 mV is shown before (control) and after exposure to 200 nM TTX. Background ionic currents and capacitance current have been subtracted, as described previously (Fu et al., 2011).

In vitro isometric force responses

Muscle contractility was measured by recording the force generated by direct electric field stimulation of whole soleus muscle suspended in a tissue bath maintained at 37 °C. The non-depolarizing neuromuscular blocker curare (0.5 μM) was added to the bath to prevent a synaptic contribution from activation of motor nerve endings. Tetanic contractions elicited by a supramaximal stimulus of 80 mA pulses at 100 Hz had a comparable rising phase, peak, and decay time for wild-type and +/Δ_Ex12 soleus (Fig. 3A). The stimulus–force relation for submaximal activation, however, had a rightward shift for +/Δ_Ex12 muscle. The current intensity required for 50% maximal force in wild-type muscle was 16.3 ± 2.2 mA, whereas for +/Δ_Ex12 muscle a larger stimulus of 24.3 ± 1.7 mA (P < 0.05) was required (Fig. 3B). This reduced excitability for +/Δ_Ex12 muscle is consistent with the reduced Na⁺ current density observed in the voltage-clamp studies above. The steady-state tetanic force elicited by a supramaximal stimulus had the same amplitude for wild-type and +/Δ_Ex12 soleus (13.5 ± 0.53 g and 12.6 ± 0.61 g, respectively, P = 0.30). Figure 3C shows a comparison of these steady-state tetanic force responses to our previous study of the NaV1.4-R669H model of HypoKPP (Wu et al., 2011), which had NaV1.4 increases (Yang et al., 1991). We tested for compensation of the NaV1.4 null allele by persistence of the TTX-R NaV1.5 isoform in fibres from +/Δ_Ex12 adult mice. The Na⁺ current was completely blocked by application of 200 nM TTX (Fig. 2B), thereby indicating that no NaV1.5 current was detectable. Taken together, these results demonstrate that the NaV1.4 Δ_Ex12 allele is non-functional and that there is no evidence for compensation by persistent expression of the NaV1.5 isoform or upregulation of the wild-type NaV1.4 allele.
Figure 3  Isometric contractile force of the soleus muscle, in vitro. (A) Force transients elicited by field stimulation with a 100 Hz  train of 80 mA current pulses applied for 400 ms. Each record is the response to a single trial. (B) Steady-state isometric force is plotted as a function of the stimulus current intensity, relative to the maximal force observed with an 80 mA stimulus. Symbols are average values for wild-type (+/+; n = 7 and +/Δ_Ex12 n = 5 soleus muscle preparations. Dashed line shows that to produce force at 50% of the maximal value, a higher stimulus current was required for (+/Δ_Ex12) compared to wild-type soleus. (C) A box plot of the steady-state force produced by 80 mA current stimulation. No difference in maximal steady-state force was observed in a comparison of wild-type (+/+; n = 12), +/Δ_Ex12 (n = 18), or NaV1.4-R669H heterozygote (+/m; n = 8) muscles; whereas the homzygous mutant (m/m; n = 6) had reduced force (P < 0.01 ANOVA). Data for NaV1.4-R669H mice reproduced from our prior report (Wu et al., 2011).

Myasthenic features for +/Δ_Ex12 muscle were also observed in the electrical responses evoked by repetitive nerve stimulation. The CMAP, which is a spatially averaged measure of muscle electrical excitation from many fibres, was recorded from the soleus muscle ex vivo, in response to stimulation of the motor nerve with a suction electrode. The CMAP amplitude in response to a single shock was comparable for wild-type and +/Δ_Ex12 muscle, 5.7 ± 1.0 mV and 4.8 ± 1.1 mV, respectively. The change in CMAP amplitude during a train of repetitive shocks to the motor nerve was used to test the fidelity of neuromuscular transmission. A decrement of the CMAP amplitude during repetitive stimulation is the hallmark of a loss of safety factor at the neuromuscular junction, as occurs in autoimmune-mediated myasthenia gravis. The CMAP amplitude for wild-type muscle was stable at low frequencies of stimulation and had a physiological decrease of ~15% at 60 and 100 Hz (Fig. 5). For +/Δ_Ex12 muscle, the response for high frequency stimulation showed a trend toward a larger decrement of ~25% (Fig. 5), although this was not statistically different from wild-type muscle. Partial block of nicotinic acetylcholine receptors with curare has been used to reveal latent defects of neuromuscular transmission in mice (Sandrock et al., 1997). In the presence of 0.5 μM curare, a pronounced decrement of CMAP amplitude was observed for repetitive stimulation at all frequencies (Fig. 5). The decrement was greater for +/Δ_Ex12 muscle compared to wild-type at frequencies of 10 Hz and greater (P < 0.05), thereby demonstrating a reduced safety factor for the coupling of nerve stimulation to muscle action potential generation in mice with a null allele for NaV1.4.

Taken together, the fatigable ‘sag’ in force during 100 Hz electric field stimulation and the latent reduction in the safety factor for triggering a muscle action potential by motor nerve stimulation provide evidence for a myasthenic...
phenotype in $+/\Delta_{\text{Ex12}}$ mice. We use the term pseudo-myasthenia because the defect resides with the intrinsic excitability of the muscle fibre for which the Na$^+$ current density is reduced, rather than classical myasthenia where the defect is in the generation of the postsynaptic end-plate potential.

The Na$_V$1.4 null allele is not sufficient to cause the hypokalaemic periodic paralysis phenotype

We have previously established protocols that demonstrate a HypoKPP phenotype in genetically modified mice that harbour human disease-associated missense mutations, Na$_V$1.4-R669H (Wu et al., 2011) and Ca$_V$1.1-R528H (Wu et al., 2012). The $+/\Delta_{\text{Ex12}}$ mice were tested with these protocols to determine whether the loss-of-function produced by a null allele for Na$_V$1.4 was sufficient to cause HypoKPP. The first test measured the susceptibility to loss of muscle force in response to a hypokalaemic challenge. Maximal tetanic force was monitored every 2 min for the isolated soleus maintained in a tissue bath. Control responses were measured in 4.7 mM K$^+$, and then the bath was exchanged with a 2 mM K$^+$ solution for 20 min. The force was normalized to the maximal tetanic contraction in control conditions and average responses are shown in Fig. 6. Wild-type soleus had only about a 10% decrease in force.
in maximal force and a similar response was observed for +/Δ_Ext12 soleus (Fig. 6A). In contrast, soleus from our mouse model of HypoKPP had a 30% loss of force for animals heterozygous for NaV1.4-R669H (+/m), and the homozygous mutant (m/m) had an 85% decrease in tetanic force. In all cases, the loss of force was reversible on return to 4.7 mM K+.

Because the phenotypic differences in force responses between wild-type and +/Δ_Ext12 soleus are revealed only at stimulation levels below saturation (cf. Figs 3B and 4C), we repeated the 2 mM K+ challenge using a submaximal stimulus intensity of 40 mA. Both wild-type and +/Δ_Ext12 soleus had a larger decline in relative force (Fig. 6A), and for +/Δ_Ext12 muscle the early peak force was followed by a sag to a lower steady-state level. The relative decrease in force for wild-type (n = 4) and +/Δ_Ext12 (n = 6) soleus, however, was not distinguishable statistically (P > 0.2).

Mutations of NaV1.4 that cause gain-of-function changes, by disrupting inactivation or enhancing activation, have been identified in families with HyperKPP wherein elevated K+ may trigger an episode of weakness (Cannon, 2015). In our NaV1.4-M1592V mouse model of HyperKPP, a 10 mM K+ challenge produces a robust decrease in tetanic force (Hayward et al., 2008). Therefore, we also tested whether exposure to 10 mM K+ would elicit a loss of force in the heterozygous null mice. Figure 6B shows that neither wild-type nor (+/Δ_Ext12) soleus had a reduction of tetanic force in 10 mM K+.

The second test for a HypoKPP phenotype was to monitor the electrical excitability of muscle in vivo during a provocative challenge by intravenous infusion of glucose and insulin. The glucose plus insulin load promotes a shift of extracellular K+ into muscle which often triggers an episode of weakness and has previously been used as a clinical diagnostic test for susceptibility to HypoKPP (Lehmann-Horn et al., 2004). Under isoflurane anaesthesia, the CMAP of the soleus and gastrocnemius muscles in response to sciatic nerve stimulation was monitored once a minute during a 60-min period of continuous infusion (0.5 ml/h). The CMAP amplitude decreased by 80% for
the $\text{Nav}_1.4$-R669H HypoKPP mouse (+/m) but was maintained for wild-type and $+/\Delta_{\text{Ex12}}$ mice (Fig. 7).

The loss-of-function caused by a null allele for $\text{Nav}_1.4$ was not sufficient to produce a HypoKPP phenotype by two criteria. Neither a loss of force for the in vitro low-$K^+$ challenge nor a reduction of CMAP amplitude from the in vivo glucose plus insulin challenge was observed.

**Discussion**

Mutations of $\text{SCN4A}$ encoding the $\alpha$-subunit of the skeletal muscle sodium channel, $\text{Nav}_1.4$, have been associated with a diverse group of muscle disorders (Lehmann-Horn et al., 2004; Horga et al., 2013; Cannon, 2015) and possibly even CNS disorders (Bergareche et al., 2015). Functional expression studies of disease-associated mutant channels have revealed gain of function, loss-of-function, or mixed effects on the influx of $Na^+$ currents (Jurkat-Rott et al., 2010).

The gain-of-function defects are encountered more commonly and are established causes of myotonia, paramyotonia, and HyperKPP. The pathologically enhanced $Na^+$ currents in these disorders are caused by disrupted inactivation [less complete (Cannon et al., 1991), slower onset (Yang et al., 1994), faster recovery (Hayward et al., 1996), depolarized shift in voltage dependence] or in some cases enhanced activation [slower deactivation (Featherstone et al., 1998), hyperpolarized shift in voltage dependence (Cummins et al., 1993)]. An unusual gain of function has been identified for mutations of $\text{Nav}_1.4$ associated with HypoKPP. All 10 mutations occur at arginine residues in S4 voltage sensor domains and create an anomalous conduction pathway—the gating pore—that allows the influx of a leakage current at the resting potential (Sokolov et al., 2007; Struyk and Cannon, 2007). Depolarization changes are less common for $\text{Nav}_1.4$ mutant channels associated with muscle disorders. Enhanced inactivation has been observed for some of the HypoKPP mutations (Struyk et al., 2000; Kuzmenkin et al., 2002), which also produced gating pore leakage currents (Struyk and Cannon, 2007), thereby creating a mixture of loss- and gain-of-function defects. Mutations causing isolated loss-of-function defects are very rare. Two patients with
myasthenic features resulting from markedly enhanced inactivation alone have been reported (Tsujino et al., 2003; Arnold et al., 2015), and a cohort of 11 patients was identified from a congenital myopathy registry that harboured recessive homozygous mutant alleles or compound heterozygous mutations that were either non-functional or had a relative loss-of-function (Zaharieva et al., 2015). We created a murine null allele for NaV1.4 that would enable us to more clearly define the consequences of a ‘pure’ loss-of-function defect on mammalian muscle excitability, potential compensatory mechanisms, and susceptibility to disease phenotypes.

The +/ delete 12 mouse is haploinsufficient for NaV1.4

The deletion of exon 12 is expected to produce a non-functional allele, but because the deletion is in-frame, raising the possibility of a functional mutant transcript or that compensation such as increased transcription of the wild-type allele or retention of the foetal isoform NaV1.5 may restore Na+ current density, we compared Na+ currents in fibres isolated from wild-type and +/ delete 12 mice. Remarkably, the Na+ current density for +/ delete 12 muscle was 54% of that observed for wild-type, and there was no detectable TTX-resistant NaV1.5 current. This lack of evidence for a compensatory increase in NaV1.4 expression at the membrane may be a consequence of the fact that the baseline CMAP amplitude was not reduced for +/ delete 12 muscle. Apparently, there is sufficient functional reserve of Na+ current density such that a 50% decrease does not prevent the generation of muscle action potentials. Another possibility is that expression levels of other channels changed to preserve muscle excitability. For either of these mechanisms, excitation–transcription coupling remains intact without a signal to boost SCN4A transcription. The robust health of the +/ delete 12 mice is consistent with this notion. Moreover relatives who carry a single non-functional SCN4A, identified from the recessive inheritance for congenital myopathy patients, are asymptomatic (Zaharieva et al., 2015).

Haploinsufficiency of some other sodium channel isoforms is associated with severe clinical phenotypes in humans. Loss-of-function mutations for the cardiac isoform NaV1.5, including null alleles incapable of forming functional channels (Deschenes et al., 2000), produce progressive cardiac conduction disease or the Brugada syndrome with plectropathic rhythm disturbances and susceptibility to ventricular tachycardia and ventricular fibrillation (Naccarelli and Antzelevitch, 2001). Mice haploinsufficient for NaV1.5 have a normal lifespan but have impaired atrioventricular conduction, increased refractoriness, and ventricular tachycardia (Papadatos et al., 2002). Similar to our NaV1.4 (+/ delete 12) mice, the Na+ current density was reduced in cardiac myocytes by 50% without evidence for compensation. A homozygous null for NaV1.5 was lethal at embryonic Day 10, whereas some NaV1.4 homozygous null mice were born live. Haploinsufficiency of a neuronal isoform, NaV1.1, causes severe myoclonic epilepsy of infancy (Dravet syndrome) which presents with early onset intractable epilepsy plus developmental delay, sleep disturbances, behavioural disorders and cognitive impairment (Claes et al., 2001). Mice haploinsufficient for NaV1.1 develop seizures at postnatal Day 21 and are susceptible to premature death (Yu et al., 2006). Homozygous NaV1.1 null mice are ataxic and die at postnatal Day 2 from inability to feed and do not survive beyond postnatal Day 17 even if manually fed. For other sodium channel isoforms expressed in brain, haploinsufficiency is better tolerated. Deletion of a single allele for NaV1.2 did not produce any change in motor behaviour or survival; whereas homozygous null NaV1.2 mice die perinatally from hypoxia with massive neuronal apoptosis (Planells-Cases et al., 2000). Haploinsufficiency of NaV1.6 was reported in a family with neuropsychological impairment (Trudeau et al., 2006), and mice heterozygous for a null mutation of NaV1.6 exhibit greater fear conditioning and social avoidance without other defects of learning (McKinney et al., 2008). A complete homozygous null of NaV1.6 in mice causes the ‘motor endplate disease’ phenotype with progressive hindlimb paresis, severe muscle atrophy, Purkinje cell degeneration and juvenile lethality (Burgess et al., 1995).

NaV1.4 loss-of-function mutations produce a spectrum of phenotypes, often with myasthenic features

The first report of an isolated NaV1.4 loss-of-function mutation was for a 20-year-old female with lifelong episodes of respiratory and bulbar weakness, ptosis, and marked fatigability of limb muscles (Tsujino et al., 2003). Repetitive nerve stimulation revealed an abnormal decrement in the CMAP, suggesting a defect of neuromuscular transmission, and led to an investigation for the possibility of congenital myasthenia. Surprisingly, there was no defect of evoked quantal release, synaptic potentials, or acetylcholine receptor kinetics, but the normal end plate potential with a depolarization to −40 mV failed to elicit an action potential (Tsujino et al., 2003). These observations implicated a possible defect of NaV1.4, and whole exon sequencing of SCN4A revealed biallelic variants S246L and V1442E. Voltage-clamp studies of channels expressed in HEK cells demonstrated a marked enhancement of fast inactivation for V1442E with a −33 mV shift to hyperpolarized potentials. This extraordinarily large shift in voltage dependence of inactivation would reduce mutant V1442E channel availability to 13% at a resting potential of −90 mV, whereas wild-type channels have 98% availability. The S246L variant had a modest −7 mV shift of inactivation and was interpreted to be a benign polymorphism. The unaffected mother and sib also carried the S246L
alle. The father was not available for testing and therefore the inheritance pattern for a myasthenia phenotype with the V1442E mutation could not be unambiguously established. Two additional cases of a myasthenic syndrome in association with NaV1.4 mutations have been reported, one in a patient homozygous for the R1457H mutation (Arnold et al., 2015) and the other homozygous for R1454W (Habbout et al., 2016). The proband with the R1457H mutation had lifelong episodes of weakness, ptosis, and external ophthalmoplegia. Repetitive nerve stimulation demonstrated a decremental CMAP response at 10 Hz and 20 Hz. The parents were third cousins who both carried a single copy of the R1457H allele, as also did three siblings, all of whom were asymptomatic. Expression studies of R1457H revealed enhanced fast inactivation with a −14 mV hyperpolarized shift and a 10-fold slowing for recovery from inactivation. The proband with the R1454W mutation had global hypotonia with poor sucking and difficulty feeding at birth, delayed motor milestones, and fluctuating weakness lasting hours to days. In later years, the fluctuations in strength worsened with bilateral facial palsy, ptosis, and ophthalmoplegia. Repetitive nerve stimulation at 3 Hz did not elicit a decremental CMAP response, although a decrease of 27% was observed after 40 min of exercise. Sodium currents recorded from heterologously expressed R1454W channels showed markedly enhanced inactivation with a −20 mV voltage shift, 10-fold slower recovery, and a 5-fold accelerated entry rate. It is noteworthy that all three missense mutations (V1442E, R1454W, and R1457H) are located in the voltage sensor of domain IV, which is strongly linked to inactivation whereas the other voltage sensors in domains I–III are coupled to channel activation. The proposed pathomechanism is that in the homozygous state, the slower recovery of these missense mutant channels cause use-dependent reduction of sodium channel availability of sufficient magnitude (e.g. 50% at 50 Hz) to produce decreased excitability and fatigue of muscle force.

A more severe recessive phenotype has recently been reported for patients with two NaV1.4 mutations when at least one allele is completely non-functional (Zaharieva et al., 2015). From a registry of congenital myopathy with neonatal hypotonia, whole exome sequencing identified 11 individuals from six families with homozygous or compound heterozygous mutations of NaV1.4. In seven cases death occurred in utero or live births did not survive the first day, and of the four surviving individuals (ages 2.5–35 years) one mutant allele was hypomorphic with reduced Na+ current density. All except the youngest are able to walk, but had neck and facial weakness, fatigue with walking, and secondary skeletal abnormalities (scoliosis, kyphosis, high arched palate). Repetitive nerve stimulation for one of four tested individuals had a decremental CMAP response at 10 Hz. Inheritance of two completely non-functional alleles was always neonatal lethal, just as occurred in our Δ_Ex12/Δ_Ex12 mice. The heterozygous relatives were asymptomatic, regardless of whether the single mutant allele was non-functional or hypomorphic.

Our NaV1.4 knock-out mouse exhibited several features in common with the clinical phenotypes of patients with severe loss-of-function mutations (Table 1). A notable finding is that NaV1.4 haploinsufficient mice and humans have preserved muscle excitability, contractility, and motor function. This outcome is remarkable in view of the fact that NaV1.4 accounts for >90% of the total Na+ current in adult skeletal muscle (Fu et al., 2011), and the mouse model had no evidence of compensatory upregulation of the normal allele. As mentioned above, haploinsufficiency is not as well tolerated for NaV1.5 in heart or NaV1.1 in brain, but is well tolerated for NaV1.2 and NaV1.6 in brain. Abnormal muscle fatigue, ptosis, and a decrement of CMAP amplitude during repetitive stimulation were features of both case reports of a congenital myasthenic syndrome associated with NaV1.4 mutations (Tsujino et al., 2003; Arnold et al., 2015). Functional expression studies showed these mutations caused abnormally enhanced inactivation that became progressively more severe during repetitive pulsing because of slower recovery from inactivation. This dynamic aspect for the loss-of-function may account for the prominence of activity-dependent fatigue and a CMAP decrement with repetitive stimulation. Conversely, a fixed deficit from a null allele had latent fatigability and latent CMAP decrement in the Δ_Ex12 mouse and was not reported for heterozygous null patients (Zaharieva et al.,

**Table 1** Spectrum of phenotypes from loss of function defects for NaV1.4

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<th>Heterozygous mutant</th>
<th>Homozygous mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/LOF (moderate)</td>
<td>+/null</td>
</tr>
<tr>
<td>+/LOF (severe)</td>
<td></td>
</tr>
<tr>
<td>+/null</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>n.d.</td>
<td></td>
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<tr>
<td>n.d.</td>
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<tr>
<td>n.d.</td>
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<tr>
<td>Subclinical fatigability</td>
<td>Latent decrement on repetitive stimulation CMAP</td>
</tr>
<tr>
<td>Fatigability, ptosis</td>
<td>Asymptomatic Latent symptoms!</td>
</tr>
<tr>
<td>Decrement on repetitive stimulation CMAP</td>
<td>Latent repetitive stimulation decrement on CMAP</td>
</tr>
<tr>
<td>Infantile hypotonia, myopathy</td>
<td>Neonatal lethal</td>
</tr>
<tr>
<td>Neonatal lethal</td>
<td></td>
</tr>
</tbody>
</table>

LOF = loss-of-function with hypomorphic preservation of Na+ current.

n.d. = not done.
Haploinsufficiency of \( \text{Na}_V 1.4 \) is not sufficient to produce a HypoKPP phenotype

Initial reports on the pathogenesis of HypoKPP caused by \( \text{Na}_V 1.4 \) missense mutations (R669H and R672H/G) focused the abnormal enhancement of inactivation (Jurkat-Rott \textit{et al.}, 2000; Struyk \textit{et al.}, 2000). These were the first reports for a loss-of-function change in disease-associated mutations of \( \text{Na}_V 1.4 \) and led to the notion that gain-of-function changes cause susceptibility to myotonia, paramyotonia, and HyperKPP, whereas loss-of-function changes cause HypoKPP. Seven years later, the anomalous gating pore leakage current was identified in these same two HypoKPP mutant channels (Sokolov \textit{et al.}, 2007; Struyk and Cannon, 2007) and subsequently all eight \( \text{Na}_V 1.4 \) HypoKPP mutations tested to date were shown to have a gating pore leakage current (Struyk \textit{et al.}, 2008; Francis \textit{et al.}, 2011). More recently, we reported a defect in the coupling of voltage-sensor displacement to channel opening as a second loss-of-function change in the two HypoKPP mutations investigated, R669H and R672G (Mi \textit{et al.}, 2014). The mixed presence of loss- and gain-of-function changes has led to a debate over whether \( \text{Na}_V 1.4 \) loss-of-function is an important contributor to susceptibility of HypoKPP attacks, and even whether the gating pore current is an epiphenomenon (Jurkat-Rott and Lehmann-Horn, 2007; Matthews \textit{et al.}, 2009; Matthews and Hanna, 2010). In contrast to our mouse model of HypoKPP (\( \text{Na}_V 1.4\)-R669H), which has a robust phenotype with dominantly inherited loss of force with low \( K^+ \) challenge \textit{in vitro}, paradoxical depolarization in low \( K^+ \), and a profound loss of CMAP amplitude and force in response to a glucose plus insulin challenge \textit{in vivo} (Wu \textit{et al.}, 2011), the +/\( \Delta \) _Ex12_ mouse had none of these features. Moreover, the +/\( \Delta \) _Ex12_ haploinsufficient state with an experimentally verified 50\% reduction of \( Na^+ \) current density produces a more severe loss of \( Na^+ \) current than the enhanced inactivation produced by a single R669H or R672H/G mutant allele in dominantly inherited HypoKPP. Therefore we conclude that a loss-of-function defect of \( \text{Na}_V 1.4 \) alone, even if as severe as haploinsufficiency, is not sufficient to cause a HypoKPP phenotype. We do agree, however, that any process that reduces \( Na^+ \) current density would exacerbate the loss of excitability caused by paradoxical depolarization in low \( K^+ \) during an attack of HypoKPP which inactivates \( \text{Na}_V 1.4 \).

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References


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