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Journal

Brain, 139(6)

ISSN

0006-8950

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Publication Date

2016-06-01

DOI

10.1093/brain/aww070

Peer reviewed

doi:10.1093/brain/aww070 BRAIN 2016: 139; 1688–1699 | 1688

BRAIN
A JOURNAL OF NEUROLOGY

Mice with an $Na_V1.4$ sodium channel null allele have latent myasthenia, without susceptibility to periodic paralysis

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Over 60 mutations of *SCN4A* encoding the $Na_V1.4$ 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 $Na_V1.4$ 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 $Na_V1.5$ isoform was detected. Mice null for $Na_V1.4$ did not survive beyond the second postnatal day. This mouse model shows remarkable preservation of muscle function and viability for haploinsufficiency of $Na_V1.4$, 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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Keywords: skeletal muscle; weakness; channelopathy; congenital myasthenic syndrome; SCN4A

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

Introduction

The voltage-activated sodium channel of skeletal muscle, $Na_V1.4$, 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 $Na_V1.4$ 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.*,

Received December 01, 2015. Revised February 09, 2016. Accepted February 18, 2016. Advance Access publication April 5, 2016

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 $Na_V1.4$ (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 $Na_V1.4$ 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 $Na_V1.4$ 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 (Tsujino *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 $Na_V1.4$ 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 $Na_V1.4$ 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 $Na_V1.4$ 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 $Na_V1.4$ 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 $Na_V1.4$-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, ENSMUST00000021056). The 5'-loxP site was 52 bp upstream from exon 12 and the 3'-loxP site was 202 bp downstream from exon 12. Heterozygous (+/$Na_V1.4$-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 $Na_V1.4$ 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'- GCCCTTCGGTCCCCAAAGCCTCTGCCAGG -3'; reverse, 5'- CCTAAGCCCCAAGCTTGTTACTAACTC -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 Ca^{2+}, followed by trituration 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% CO_2 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

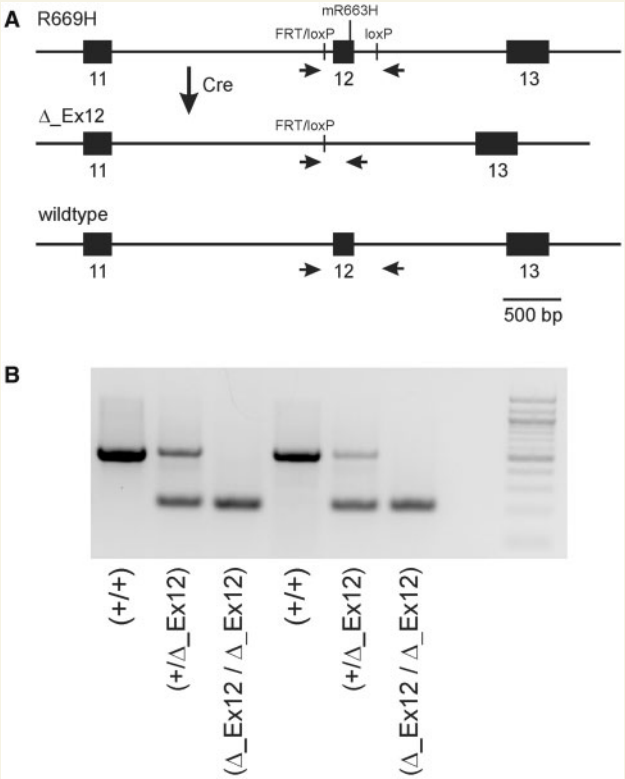

Figure 1 Generation of the $Na_V1.4$ null allele. (**A**) A knock-in mutation for mR663H in exon 12 (denoted R669H in reference to the human mutation) was previously generated by homologous recombination (Wu *et al.*, 2011) and also contained loxP sites flanking exon 12 (*top*). Crossing with a Cre expressing line deleted exon 12 and 254 bp of intronic DNA. (**B**) Genotyping by PCR amplification produced a 525 bp product from the wild-type allele and a 212 bp product from the Cre-deleted allele.

400 mM glycerol for 1 h to improve the voltage-clamp control of membrane potential. Currents were recorded in a Cl^--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 μM 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).

In vitro contraction studies

Isometric force was measured for the isolated soleus muscle that was suspended between a fixed post and a force transducer (Fort25, World Precision Instruments, Inc.). The muscle was immersed in a tissue bath maintained at 37 °C and continuously bubbled with 95% O_2 and 5% CO_2. The bath contained (in mM) 118 NaCl, 4.75 KCl, 1.18 $MgSO_4$, 2.5 CaCl, 1.18 NaH_2PO_4, 24.8 $NaHCO_3$, 10 glucose, and 158 nM (20 U/l) insulin. For tests of intrinsic contractility (Figs 3 and 4), the muscle was stimulated directly by parallel wire electrodes aligned perpendicular to the fibre axis. D-tubocurarine (0.5 μM) was added to block muscle activation from motor nerve endings. Tetanic stimulation at 100 Hz × 40 pulses of 1-ms duration was applied from an isolated current source of variable intensity (0–100 mA; Model A385, World Precision Inst).

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 μM, 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 α-subunit of $Na_V1.4$ (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 ($Na_V1.4$-R669H). The targeting construct also contained intronic LoxP sites that flanked exon 12. Heterozygous (+/$Na_V1.4$-R669H) mice were crossed with a Cre recombinase expressing line [$Meox2^{tm1(cre)Sor}$] to produce exon 12-deleted heterozygotes (+/Δ_Ex12). Confirmation by genotyping was performed by PCR amplification of genomic DNA with a set of intronic primers flanking exon 12 (Fig. 1B). The wild-type allele produced a 525 bp amplimer, whereas the Cre deleted allele produced a 212 bp amplimer.

Heterozygotes (+/Δ_Ex12) were viable, fed and gained weight normally, had no gross motor deficits, and bred successfully. Crosses of +/Δ_Ex12 and wild-type mice yielded heterozygous (+/Δ_Ex12) animals at the expected

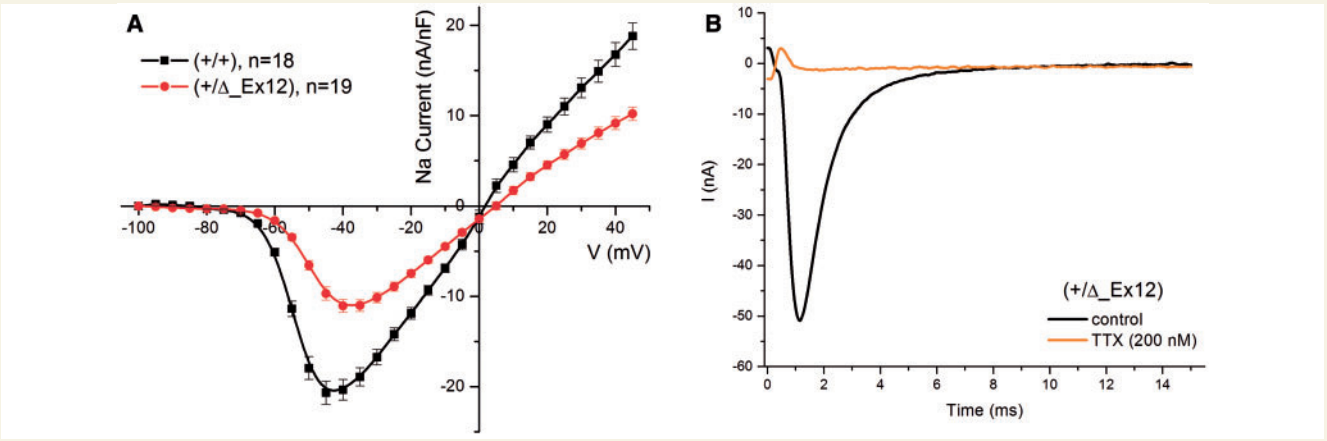

Figure 2 Sodium current density is reduced in +/Δ_Ex12 muscle. (**A**) Peak Na^+ current amplitude normalized to whole-cell capacitance is plotted as a function of the test depolarization from a holding potential of −100 mV. (**B**) Sodium current recorded from a +/Δ_Ex12 fibre in response to a test 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).

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 S2 through the first seven residues 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 membrane potential in Fig. 2A and shows the amplitude was reduced by about a factor of 2 in +/Δ_Ex12 mice, without any change in the voltage dependence of activation. The maximal inward Na^+ current density for wild-type muscle was -20.3 ± 1.14 nA/nF, while in +/Δ_Ex12 fibres the maximum was -11.0 ± 0.71 nA/nF ($P < 0.001$). Two sodium channel isoforms are expressed in mammalian skeletal muscle. The tetrodotoxin-resistant (TTX-R) $Na_V1.5$ isoform predominates during foetal development and then declines at birth as the expression of the adult isoform $Na_V1.4$ increases (Yang *et al.*, 1991). We tested for compensation of the $Na_V1.4$ null allele by persistence of the TTX-R $Na_V1.5$ 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 $Na_V1.5$ current was detectable. Taken together, these results demonstrate that the $Na_V1.4$ Δ_Ex12 allele is non-functional and that there is no evidence for compensation by persistent expression of the $Na_V1.5$ isoform or upregulation of the wild-type $Na_V1.4$ allele.

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 muscle (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 $Na_V1.4$-R669H model of HypoKPP (Wu *et al.*, 2011), which had

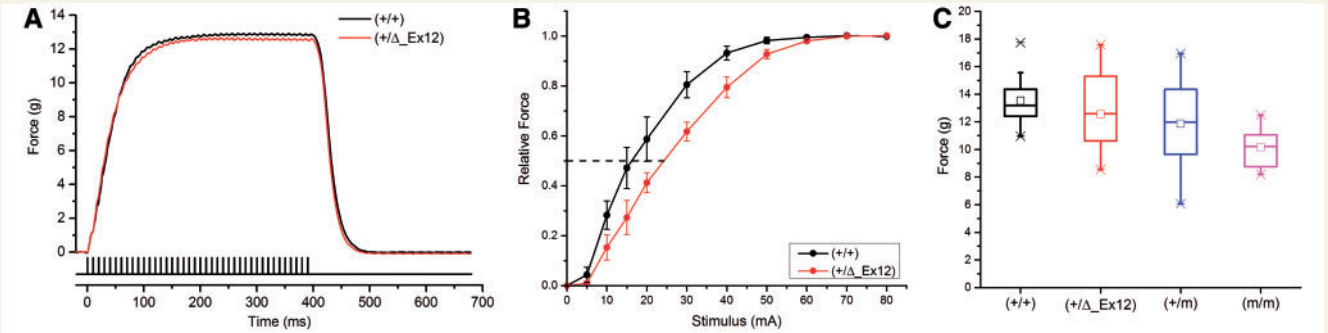

Figure 3 Isometric contractile force of the soleus muscle, *in vitro*. (**A**) Force transients elicited by field stimulation with a 100 Hz train of 80 mA current pulses applied for 400 ms. Each record is the response to a single trial. (**B**) Steady-state isometric force is plotted as a function of 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 $Na_V1.4$-R669H heterozygote (+/m; $n = 8$) muscles; whereas the homozygous mutant (m/m; $n = 6$) had reduced force ($P < 0.01$ ANOVA). Data for $Na_V1.4$-R669H mice reproduced from our prior report (Wu *et al.*, 2011).

a reduced maximal force for homozygous mutants, $P < 0.01$.

Pseudo-myasthenia of +/Δ_Ex12 muscle

The force transients elicited by direct electrical field stimulation at submaximal current intensity had a prominent 'sag' after the early peak for +/Δ_Ex12 muscle that was not present in wild-type muscle. Typical responses for a sequence of force transients as the stimulus intensity was increased are shown in Fig. 4A. Each trace is a single trial, with the set of responses displayed for a single muscle as the current was increased from 10 mA to 40 mA. The amplitude of the decline in force, relative to the early peak, was larger for responses at low stimulus intensity (Fig. 4B), and was much larger for soleus from +/Δ_Ex12 mice than wild-type. The more prominent decline in force for +/Δ_Ex12 soleus was not attributable to the right shift of the stimulus–force curve (Fig. 3B) because a comparable right shift for the wild-type responses in Fig. 4B (dotted line) does not overlap with the responses observed in +/Δ_Ex12 muscle. An alternative method to demonstrate that the more prominent sag for +/Δ_Ex12 muscle is not attributable to a bias produced by the shift in the stimulus-force relation is shown in Fig. 4C. Each symbol shows the relative decline in force from the early peak within one trial, now plotted against the amplitude of the early peak relative to the maximum early peak force measured at the highest stimulus intensity. A prominent sag (>0.2 relative decline) was observed in +/Δ_Ex12 muscle even for trials in which the early peak force was 0.6 of the maximal peak value. For wild-type muscle, however, the amplitude of the decline was always <0.2 and became very small for all trials where the early peak force was >0.4 of the maximum.

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 $Na_V1.4$.

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

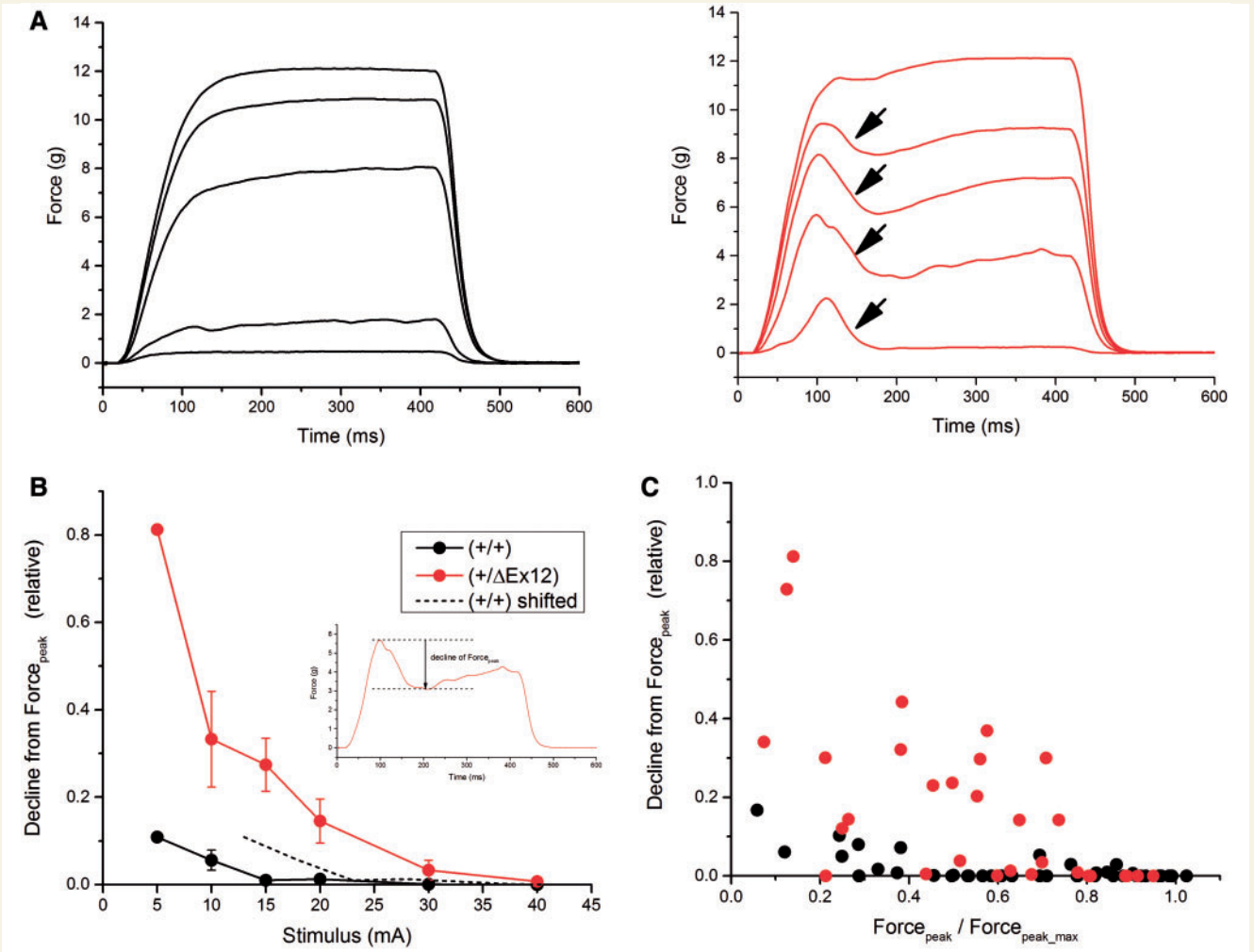

Figure 4 Pseudo-myasthenic decline in force for +/Δ_Ex12 soleus. (**A**) Force transients elicited by field stimulation over a range of submaximal current stimulus intensities. Each trace is a single trial [black for wild-type (+/+), red for +/Δ_Ex12] in response to a 100 Hz pulse train of 400-ms duration. Current intensity was increased from 10, 15, 20, 30, to 40 mA (smallest to greatest response). A prominent decline in force occurred ~100 ms from stimulus onset (arrows) for +/Δ_Ex12 soleus. (**B**) The amplitude of the decline in force, relative to the early peak (see inset), is shown as a function of stimulus intensity. The decline was much larger for (+/Δ_Ex12) (n = 6), than for wild-type (+/+) soleus (n = 8), even if a compensatory rightward shift was introduced (dashed line) to offset the difference stimulus-force relationship shown in Fig. 3B. (**C**) The relative decline in force is plotted as a function of the early peak amplitude, normalized to the maximum observed for an 80 mA stimulus. This transformation shows the decline in force for +/Δ_Ex12 soleus was substantial even when the force level was >50% of the maximal value. Each symbol is the response for a single trial.

phenotype in +/Δ_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 $Na_V1.4$ 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, $Na_V1.4$-R669H (Wu *et al.*, 2011) and $Ca_V1.1$-R528H (Wu *et al.*, 2012). The +/Δ_Ex12 mice were tested with these protocols to determine whether the loss-of-function produced by a null allele for $Na_V1.4$ 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

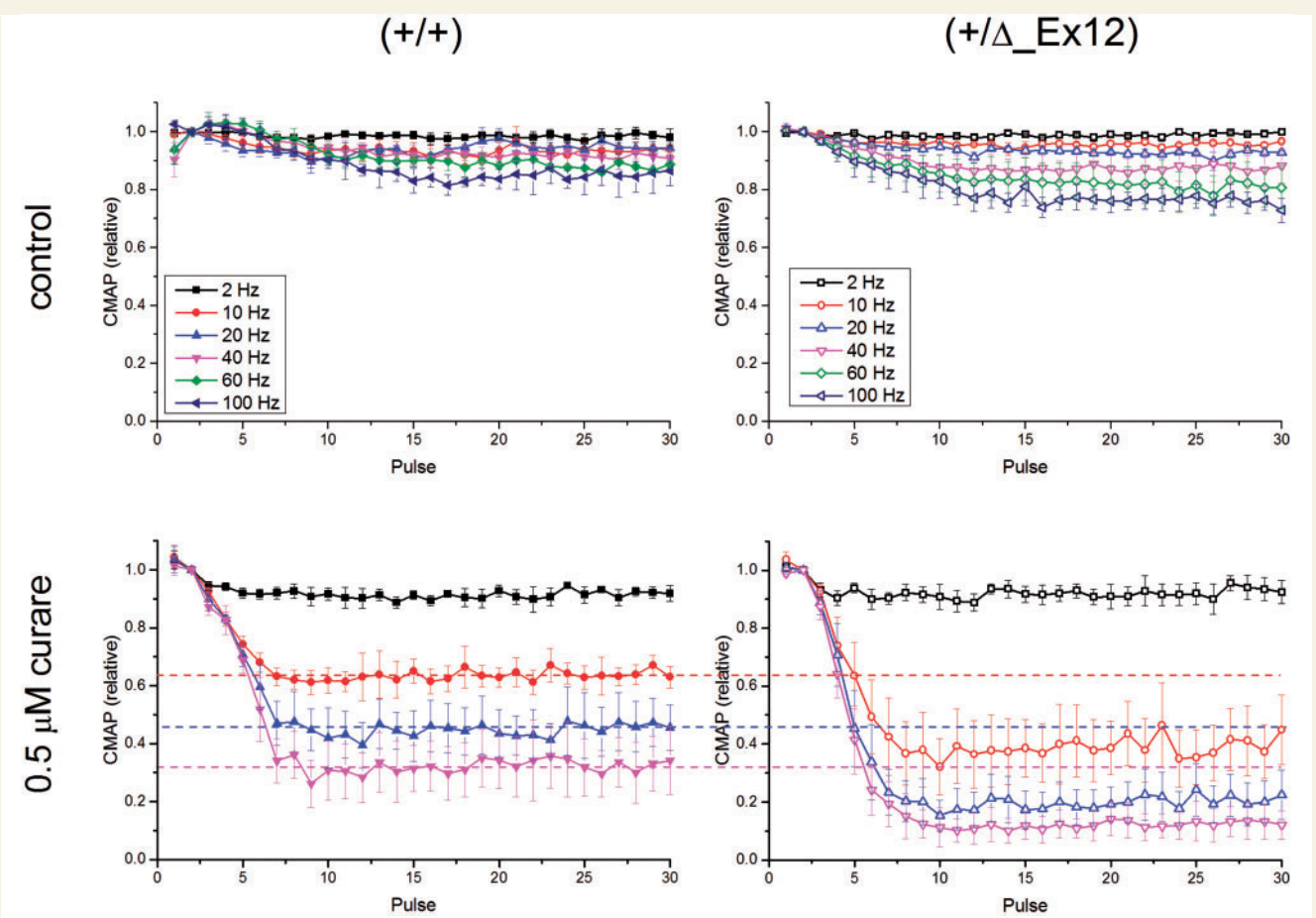

Figure 5 Pseudo-myasthenic CMAP response to repetitive nerve stimulation. The relative amplitude of the CMAP during repetitive nerve stimulation is plotted as a function of pulse number. At baseline (control, *top row*) a small decrease in CMAP amplitude occurred at higher frequencies of stimulation for wild-type (+/+) soleus ($n = 3$) and the decrease tended to be slightly larger for +/Δ_Ex12 soleus ($n = 3$), but was not statistically different. Partial block of nicotinic acetylcholine receptors with curare (*bottom row*) revealed a latent defect of neuromuscular transmission shown by the more prominent decline of +/Δ_Ex12 CMAP during repetitive nerve stimulation.

in maximal force and a similar response was observed for +/Δ_Ex12 soleus (Fig. 6A). In contrast, soleus from our mouse model of HypoKPP had a 30% loss of force for animals heterozygous for $Na_V1.4$-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_Ex12 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_Ex12 soleus had a larger decline in relative force (Fig. 6A), and for +/Δ_Ex12 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_Ex12 ($n = 6$) soleus, however, was not distinguishable statistically ($P > 0.2$).

Mutations of $Na_V1.4$ 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 $Na_V1.4$-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_Ex12) 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

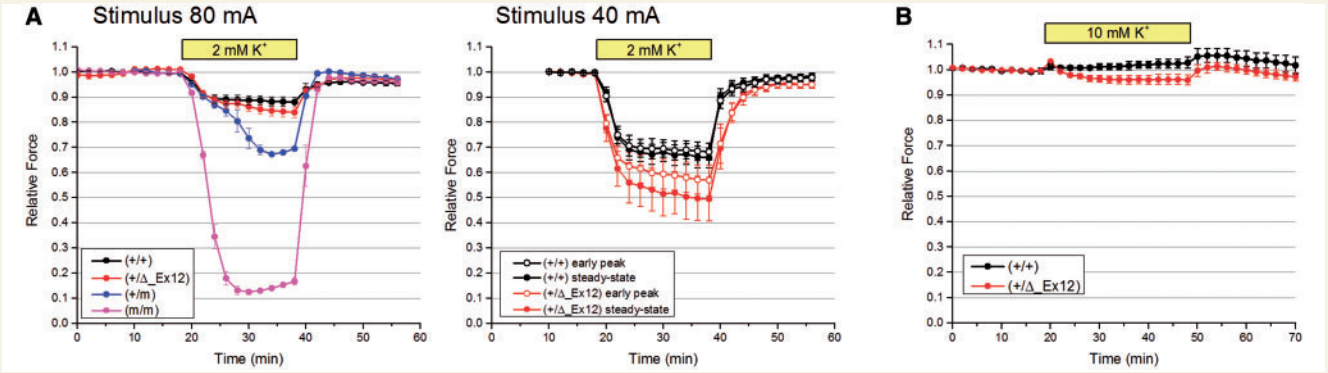

Figure 6 Potassium challenge during *in vitro* contraction. (**A**) Tetanic force is normalized to the control response in 4.7 mM K^+, and a hypokalaemic challenge of 2 mM was applied for 20 min. With a supramaximal stimulus intensity of 80 mA (*left*), soleus from wild-type (+/+) and +/Δ_Ex12 had a small decrease of 10% to 15% that was indistinguishable. Responses from HypoKPP soleus muscle with the $Na_V1.4$-R669H mutation, however, exhibited larger decrease of 30% decrease for heterozygous (+/m) and 85% for homozygous mutants (m/m); data reproduced from our previous study (Wu *et al.*, 2011). With a submaximal stimulus intensity of 40 mA (*right*), a larger decrease in force occurred for both wild-type (+/+) and +/Δ_Ex12 soleus in 2 mM K^+. In addition, the early peak force for +/Δ_Ex12 muscle (open symbols, red) was followed by a sag to a lower steady state (filled symbol); whereas no detectable sag was observed from wild-type muscle (black). (**B**) A hyperkalemic challenge with 10 mM K^+ did not elicit a reduction of tetanic force for wild-type or +/Δ_Ex12 soleus muscle.

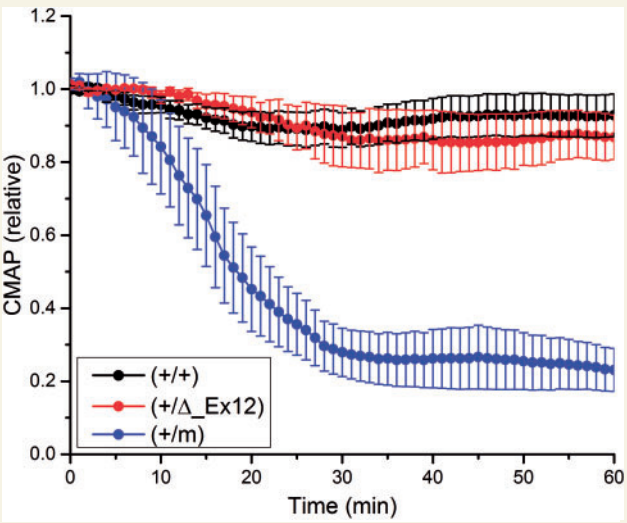

Figure 7 CMAP during a glucose plus insulin challenge. Muscle excitability *in vivo* was monitored by the amplitude of the gastrocnemius and soleus CMAP elicited by stimulation of the sciatic nerve. A continuous infusion of glucose plus insulin started at time 0 and consistently produced a decrease in CMAP for HypoKPP susceptible mice [$Na_V1.4$-R669H heterozygotes, (+/m) reproduced from our prior study (Wu *et al.*, 2011)], but not for controls, wild-type (+/+) or +/Δ_Ex12.

the $Na_V1.4$-R669H HypoKPP mouse (+/m) but was maintained for wild-type and +/Δ_Ex12 mice (Fig. 7).

The loss-of-function caused by a null allele for $Na_V1.4$ 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 *SCN4A* encoding the α-subunit of the skeletal muscle sodium channel, $Na_V1.4$, 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 $Na_V1.4$ 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). Loss-of-function changes are less common for $Na_V1.4$ 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 $Na_V1.4$ 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 +/Δ_Ex12 mouse is haploinsufficient for $Na_V1.4$

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 $Na_V1.5$ may restore Na^+ current density, we compared Na^+ currents in fibres isolated from wild-type and +/Δ_Ex12 mice. Remarkably, the Na^+ current density for +/Δ_Ex12 muscle was 54% of that observed for wild-type, and there was no detectable TTX-resistant $Na_V1.5$ current. This lack of evidence for a compensatory increase in $Na_V1.4$ expression at the membrane may be a consequence of the fact that the baseline CMAP amplitude was not reduced for +/Δ_Ex12 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 +/Δ_Ex12 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 $Na_V1.5$, including null alleles incapable of forming functional channels (Deschenes *et al.*, 2000), produce progressive cardiac conduction disease or the Brugada syndrome with pleotropic rhythm disturbances and susceptibility to ventricular tachycardia and ventricular fibrillation (Naccarelli and Antzelevitch, 2001). Mice haploinsufficient for $Na_V1.5$ have a normal lifespan but have impaired atrioventricular conduction, increased refractoriness, and ventricular tachycardia (Papadatos *et al.*, 2002). Similar to our $Na_V1.4$ (+/Δ_Ex12) mice, the Na^+ current density was reduced in cardiac myocytes by 50% without evidence for compensation. A homozygous null for $Na_V1.5$ was lethal at embryonic Day 10, whereas some $Na_V1.4$ homozygous null mice were born live. Haploinsufficiency of a neuronal isoform, $Na_V1.1$, 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 $Na_V1.1$ develop seizures at postnatal Day 21 and are susceptible to premature death (Yu *et al.*, 2006). Homozygous $Na_V1.1$ 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 $Na_V1.2$ did not produce any change in motor behaviour or survival; whereas homozygous null $Na_V1.2$ mice die perinatally from hypoxia with massive neuronal apoptosis (Planells-Cases *et al.*, 2000). Haploinsufficiency of $Na_V1.6$ was reported in a family with neuropsychological impairment (Trudeau *et al.*, 2006), and mice heterozygous for a null mutation of $Na_V1.6$ exhibit greater fear conditioning and social avoidance without other defects of learning (McKinney *et al.*, 2008). A complete homozygous null of $Na_V1.6$ 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).

$Na_V1.4$ loss-of-function mutations produce a spectrum of phenotypes, often with myasthenic features

The first report of an isolated $Na_V1.4$ 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 $Na_V1.4$, 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

Table 1 Spectrum of phenotypes from loss of function defects for $Na_V1.4$

	Heterozygous mutant			Homozygous mutant		
	+/LOF (moderate)	+/LOF (severe)	+/null	LOF/LOF (moderate)	null/LOF (moderate)	null/null
Mouse	n.d.	n.d.	Subclinical fatigability Latent decrement on repetitive stimulation CMAP	n.d.	n.d	Neonatal lethal
Human	Asymptomatic	Fatigability, ptosis Decrement on repetitive stimulation CMAP	Asymptomatic Latent symptoms? Latent repetitive stimulation decrement on CMAP?	Fatigability, ptosis Decrement on repetitive stimulation CMAP	Infantile hypotonia, myopathy	Neonatal lethal

LOF = loss-of-function with hypomorphic preservation of Na^+ current.
n.d. = not done.

allele. 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 $Na_V1.4$ 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 ophthalmoparesis. 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 $Na_V1.4$ 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 $Na_V1.4$. 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, fatigability 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 $Na_V1.4$ 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 $Na_V1.4$ haploinsufficient mice and humans have preserved muscle excitability, contractility, and motor function. This outcome is remarkable in view of the fact that $Na_V1.4$ 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 $Na_V1.5$ in heart or $Na_V1.1$ in brain, but is well tolerated for $Na_V1.2$ and $Na_V1.6$ 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 $Na_V1.4$ 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.*,

2015). This suggests compensation for a static deficiency of $Na_V1.4$ produced by a null allele results in a subclinical or latent phenotype, whereas the transient worsening by exaggerated use-dependent inactivation manifests as a myasthenic phenotype (Table 1). Support for this proposal is given by the normal amplitude of the CMAP at baseline for +/Δ_Ex12 mice and congenital myopathy patients with a single copy of a completely non-functional mutant allele (Zaharieva *et al.*, 2015). It is also noteworthy that with $Na_V1.4$ mutations the CMAP decrement occurred only at high frequencies and prolonged durations of stimulation (10 Hz with preconditioning or prolonged 10 Hz pulse trains, or >20Hz) whereas the classical CMAP decrement for myasthenia gravis occurs within the second to fifth stimulus at 2 Hz or 3 Hz. This difference likely reflects two distinct mechanisms. In myasthenia gravis the reduced safety factor of neuromuscular transmission is revealed by rapid depletion of the readily releasable pool of presynaptic vesicles within the first few depolarizations, which in the setting of a disrupted postsynaptic endplate causes intermittent failure of muscle action potentials. With the $Na_V1.4$ mutations, however, a high pulse frequency and longer duration are required to cause a sufficient number of mutant $Na_V1.4$ channels to be trapped in the inactive state and decrease the reliability of action potential generation. Moreover, the anomalous pseudo-myasthenic fatigue of muscle force was observed with direct field stimulation of +/Δ_Ex12 muscle that bypassed the neuromuscular junction (Fig. 4).

Haploinsufficiency of $Na_V1.4$ is not sufficient to produce a HypoKPP phenotype

Initial reports on the pathogenesis of HypoKPP caused by $Na_V1.4$ missense mutations (R669H and R672H/G) focused the abnormal enhancement of inactivation (Jurkat-Rott *et al.*, 2000; Struyk *et al.*, 2000). These were the first reports for a loss-of-function change in disease-associated mutations of $Na_V1.4$ 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 gaiting pore leakage current was identified in these same two HypoKPP mutant channels (Sokolov *et al.*, 2007; Struyk and Cannon, 2007) and subsequently all eight $Na_V1.4$ HypoKPP mutations tested to date were shown to have a gating pore leakage current (Struyk *et al.*, 2008; Francis *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 *et al.*, 2014). The mixed presence of loss- and gain-of-function changes has led to a debate over whether $Na_V1.4$ 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 *et al.*, 2009; Matthews and Hanna, 2010). In contrast to our mouse model of HypoKPP ($Na_V1.4$-R669H), which has a robust phenotype with dominantly inherited loss of force with low K^+ challenge *in vitro*, paradoxical depolarization in low K^+, and a profound loss of CMAP amplitude and force in response to a glucose plus insulin challenge *in vivo* (Wu *et al.*, 2011), the +/Δ_Ex12 mouse had none of these features. Moreover, the +/Δ_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 $Na_V1.4$ 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 $Na_V1.4$.

Acknowledgement

We thank Hillery Gray for care of the animals and genotyping.

Funding

This work was supported by Grant AR-42703 (S.C.C.) from NIAMS of the National Institutes of Health.

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