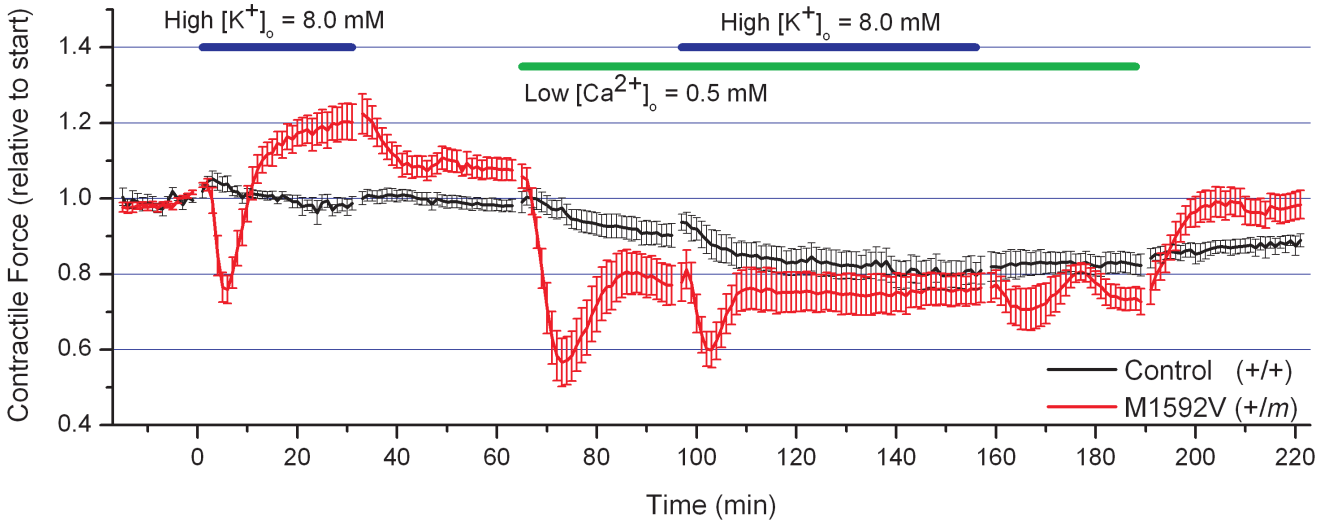
SUPPLEMENTAL DATA

Supplemental Figure 1. Increased susceptibility to weakness was produced by raising $[K^+]_o$ or lowering $[Ca^{2+}]_o$ for mutant (+/*m*) compared to normal (+/+) EDL muscle. Isolated EDL muscles from (+/*m*) mice (n=9, 10.6 ± 2.2 mo old) or (+/+) mice (n=4, 11.5 ± 1.8 mo old) were equilibrated for >30 min at 25°C in the bath solution containing 4 mM $[K^+]$ and 2 mM $[Ca^{2+}]$. Tetanic stimuli (1-ms current pulses for 100 ms at 100 Hz) were applied every 1 min, and the peak tetanic response (mean ± SE) for (+/*m*) and (+/+) muscle is shown normalized to the starting values, 13.4 ± 1.7 g for (+/*m*) compared to 21.0 ± 3.1 g for (+/+) muscle ($p<0.05$). The bath was exchanged with an otherwise identical solution containing 8 mM $[K^+]$ during the indicated period, which produced a transient loss of force by 27% for mutant muscle compared to control at 6 min ($p=0.0005$) followed by a rebound increase above the starting force by 20% at 30 min ($p=0.01$). Lowering of $[Ca^{2+}]_o$ to 0.5 mM produced transient impairment of mutant muscle contractility by 42% compared to control at 73 min ($p=0.002$). Raising $[K^+]_o$ in the presence of low $[Ca^{2+}]_o$ transiently reduced the force by >20% for mutant compared to control at 102 min ($p=0.002$). The rebound above the starting force was not observed in the comparable experiment of Figure 6B, which most likely relates to differences between the experimental conditions. The muscles of Figure 6 were i) pre-incubated in a high-Ca^{2+}-containing buffer and then equilibrated in buffer containing 1.3 mM $[Ca^{2+}]$ before applying the test condition, ii) stimulated every 3 min with 0.5-ms pulses rather than every 1 min with 1-ms pulses to reduce possible activity-related induction of the Na^+/K^+ pumps, and iii) not pre-incubated with insulin.

Supplemental Figure 1



Supplemental Videos 1 and 2. Electromyography of normal (+/+) and mutant (+/*m*) mouse hindlimb muscle. Normal (+/+) mouse muscle elicited only transiently increased activity upon needle electrode repositioning **(Supplemental Video 1)**. Muscle from a heterozygous mutant (+/*m*) sibling exhibited frequent runs of myotonia superimposed upon a high background level of activity **(Supplemental Video 2)**.

Supplemental Videos 3 and 4. Motor activity of (+/*m*) and (*m/m*) mutant $Na_V1.4$ mice. When held by the tail, the 2.8 mo old homozygous mutant (*m/m*) mouse displayed an abnormal hindlimb clasping behavior **(Supplemental Video 3)**. The smaller (*m/m*) mouse on the left exhibited gross hindlimb weakness compared to normal locomotor activity of the heterozygous (+/*m*) sibling mouse on the right **(Supplemental Video 4)**.

Supplemental Methods

Cloning of the mouse $Na_V1.4$ gene and construction of the mutant targeting vector for homologous recombination. A unique 3'-untranslated region (UTR) hybridization probe, mSkE24, identified genomic clones containing mouse $Na_V1.4$ ($mNa_V1.4$) sequence from a strain 129/Sv mouse genomic DNA library. The mSkE24 probe was obtained by polymerase chain reaction (PCR) amplification of strain 129 mouse genomic DNA using two oligonucleotide primers derived from the rat $Na_V1.4$ ($rNa_V1.4$) sequence (GenBank M26643). The upstream primer, rSk5946F (5'-CCA GGG GTC AAA GAG TCT CTT G-3'), from the 3' end of the $rNa_V1.4$ coding region, was identical to bases 667-688 of a partial sequence for $mNa_V1.4$ (GenBank S71139) (1). The downstream primer, rSk6596R (5'-CAG CTG GCA GGA CCC

AGC CTG T-3'), was selected from a region of the $Na_V1.4$ 3'-UTR that shared complete homology between known rat and human $Na_V1.4$ genes. The 630 bp PCR product amplified from mouse DNA was gel purified, labeled with ^{32}P-dGTP by random priming, and hybridized to Southern blots of strain 129 mouse genomic DNA. The probe hybridized to unique DNA fragments obtained from digestion with *Bgl*II (6.8 kb fragment), *Eco*RI (11.0 kb), *Kpn*I (6.3 kb), *Nhe*I (4.5 kb), or *Sac*I (8.9 kb) enzymes, indicating specificity for a single $mNa_V1.4$ gene (data not shown).

The mSkE24 probe hybridized strongly to three genomic clones (50E15, 135P7, and 294M11) from the Citb/CJ7 mouse BAC library (2) obtained from 129/Sv mouse ES cell line CJ7 (Research Genetics, Huntsville, AL). Clone 50E15, containing genomic sequence and the pBeloBAC11 vector (GenBank U51113), was digested with either *Eco*RI, *Hin*dIII, *Kpn*I, or *Sac*I, and restriction fragments were shotgun cloned into the plasmid vector pZErO-2.1 (Invitrogen). Overlapping clones containing the 3'UTR probe sequence were identified by colony hybridization of the labeled mSkE24 probe. Insert sizes for clones mSk.RI, mSk.H3, mSk.K6, and mSk.S1 were 11.0 kb for the *Eco*RI fragment, 2.6 kb for th *Hin*dIII fragment, 6.3 kb for the *Kpn*I fragment, and 8.9 for the *Sac*I fragment, respectively. Restriction mapping and partial sequencing of these clones revealed exon/intron boundaries identical to that of the human $Na_V1.4$ gene.

The targeting vector included a 5.8 kb long arm (*Sac*I to *Avr*II, spanning exons 17-23) and a 4.8 kb short arm (*Avr*II to *Eco*RI, containing exon 24 including the 3'UTR) of sequence homologous to the $mNa_V1.4$ gene. To construct the vector, two point mutations were introduced into a 1.8 kb *Bsm*BI / *Sse*8387I fragment by the megaprimer PCR method (reviewed in (3)). The first mutation was a missense A→G transition that substituted Val for Met in the protein at a

position corresponding to residue 1592 in the human gene (Figure 1D) and also removed an *Nsp*I site present in the wild type sequence. The second mutation was a silent C→T substitution located four nucleotides 5' to the first mutation (Figure 1D) that introduced a unique *Hpa*I restriction site for subsequent screening by Southern blotting or PCR methods. PCR of the mSk.S1 template with primers mSk939R (5'-TCC ATT TCC CCA GAG TCA CCC AG-3') and M1585VF (5'-CTC CTT CCT CAT CGT GGT **T**AA C**G**T GTA CAT TGC CAT CAT CC-3') generated a 0.3 kb megaprimer containing the mutations. A long PCR reaction incorporating the mutagenic megaprimer and the upstream primer mSk700F (5'-CCA AGC ACA ACA AGT GTC TC-3') produced a 2.3 kb product that was digested with *Bsm*BI and *Sse*8387I to yield a 1.8 kb mutagenic cassette. This DNA was ligated into the 8.9 kb *Sac*I-fragment in mSk.S1 (first cloning step, MV1.2R clone), and the entire cassette and flanking region were sequenced to verify incorporation of only the desired mutations.

To increase the length of homologous sequence in the short arm of the targeting vector, a 1.8 kb *Eco*RI fragment from mSk.K6 was ligated into *Eco*RI-digested targeting vector DNA (second cloning step, 1.2R/K6-4 clone). The PGKneo gene (4) flanked on each end by a 42-bp LoxP sequence (5'-CAA CAA CTT CGT ATA ATG TAT GCT ATA CGA AGT TAT CAG TAC-3') was liberated by double partial digestion with *Spe*I and *Xba*I from plasmid p1338 (GenBank AF335419.1), a gift from the lab of T. J. Ley (Washington University, St. Louis, MO). This 1.6 kb LoxP-PGKneo-LoxP cassette was ligated, in opposite transcriptional orientation to that of the $mNa_V1.4$ gene, into intron 23 at the *Avr*II site of a 5.2-kb *Xba*I-fragment obtained from the targeting construct from step one (third cloning step, 6.11 clone). The resulting 3.4 kb mutagenic cassette containing LoxP-PGKneo-LoxP was liberated by digestion with *Bsm*BI (partial digest) plus *Sse*8387I and then ligated into the cassette region of the

targeting vector from step two (fourth cloning step, mSk.ES3 clone). The entire 3.4 kb cassette and flanking region within the final 15.4 kb targeting vector mSk.ES3 were sequenced to verify the mutations and the LoxP-PGKneo-LoxP insertion sequence.

Generation of Met1592Val mNa$_V$1.4 knock-in mice. mSk.ES3 was linearized within the pZErO-2.1 backbone by digestion with *Sna*BI, extracted with chloroform, precipitated under ethanol, and resuspended in TE buffer. The DNA was electroporated into mouse 129/SvJae J1 ES cells at the Massachusetts General Hospital Knock-in Mouse Core facility under the direction of Dr. En Li. After selection with G418, 15 out of 200 clones were identified as correctly targeted clones by Southern blot analysis using an external hybridization probe. Chimeric mice were produced by injection of cells from these targeted clones into blastocysts at the Knock-in Core facility. Male chimeras were mated with C57BL/6J females, and offspring were screened for germline integration of the mutations and LoxP-PGKneo-LoxP sequence. Offspring have been bred into the C57BL/6J background for >9 generations, and the line in this background is designated B6.129S4-*Scn4a*^{tm1Ljh}, according to the preferred Jackson Laboratory nomenclature.

Genotyping. Each 50 µl PCR reaction contained 10 mM Tris-HCl (pH 9.2), 25 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 30 pmol of each oligonucleotide primer, 1.25 U Taq and 0.6 U Pfu DNA polymerases, and 50 ng of genomic DNA template. Denaturation was at 95^{o}C for 30 s, annealing at 62^{o}C for 60 s, and extension at 72°C for 90 s for 35 cycles. For the genotyping in Figure 1D, the primers were (a) mSk862F = 5'-AGC CTC TGT TTG TCT CTG TAG GTC TCG CAC-3' and (b) mSk998R = 5'-CTG AGC ACA ATC TCC ATT TCC CTC AGC-3'. 10 µl of the PCR product was mixed with 10 µl of a digestion buffer (pH 7.9) containing 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, and 1.25 U of either *Hpa*I or *Nsp*I restriction enzyme and incubated overnight at 37°C followed by separation

on a 1.5% agarose/TAE gel. An improved primer pair for genotyping, mSk9009F = 5'-TCG CCT ACG TCA AGA AAG AGT C-3' and mSk10157R = 5'-ACC CTG AGC ACA ATC TCC ATT T-3', produces a 1.15 kb product using standard PCR protocols.

Muscle staining. Quadriceps, hamstring, tibialis anterior, gastrocnemius, and soleus muscles were dissected, flash frozen in isopentane, and sectioned (10 μm) using a Cryostat. For hematoxylin and eosin staining, sections were immersed in Harris' hematoxylin for 1 min, rinsed in tap water, immersed in 1% eosin Y stain for 1-2 min, rinsed, dehydrated in ascending alcohol solutions, cleared with xylene, and mounted with Permount. For succinate dehydrogenase (SDH) staining, sections were incubated for 60 min at 37°C in a solution containing 0.2 M sodium phosphate buffer (pH 7.6), 0.1 M sodium succinate, and 1.2 mM nitro blue tetrazolium (Sigma). Sections were washed with water three times, exchanged in an acetone series, rinsed in water, and mounted in an aqueous mounting medium. For immunostaining using myosin-specific monoclonal antibodies, 10 μm frozen sections were incubated with blocking solution containing 5% horse serum and 0.1% BSA in PBS (pH 7.4) for 30 min (all incubations at room temperature). The sections were briefly rinsed twice and then washed for 5 min with 0.1% BSA in PBS. Sections were incubated with undiluted primary antibody A4.74 for 60 min and then rinsed three times with 0.1% BSA in PBS as above. Sections were then incubated with secondary antibody (biotinylated anti-mouse, 1: 200 dilution into 1% horse serum and 0.1% BSA in PBS) for 60 min and rinsed three times with PBS. Sections were stained using a VectaStain Elite ABC kit (Vector Laboratories) according to the manufacturer's instructions, dehydrated, and imaged by light microscopy.

References

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