Title: Differential encoding of mammalian proprioception by voltage-gated sodium channels

Short Title: Encoding of proprioception by sodium channels

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Abstract

Animals that require purposeful movement for survival are endowed with mechanosensory neurons called proprioceptors that provide essential sensory feedback from muscles and joints to spinal cord circuits, which modulates motor output. Despite the essential nature of proprioceptive signaling in daily life, the mechanisms governing proprioceptor activity are poorly understood. Here, we have identified distinct and nonredundant roles for two voltage-gated sodium channels (Na_Vs), $Na_V1.1$ and $Na_V1.6$, in mammalian proprioception. Deletion of $Na_V1.6$ in somatosensory neurons ($Na_V1.6^{cKO}$ mice) causes severe motor deficits accompanied by complete loss of proprioceptive transmission, which contrasts with our previous findings using similar mouse models to target $Na_V1.1$ ($Na_V1.1^{cKO}$). In $Na_V1.6^{cKO}$ animals, loss of proprioceptive feedback caused non-cell-autonomous impairments in proprioceptor end-organs and skeletal muscle that were absent in $Na_V1.1^{cKO}$ mice. We attribute the differential contribution of $Na_V1.1$ and $Na_V1.6$ in proprioceptor function to distinct cellular localization patterns. Collectively, these data provide the first evidence that Na_V subtypes uniquely shape neurotransmission within a somatosensory modality.

42   **Teaser**

43   Voltage gated sodium channels differentially encode mammalian proprioception via distinct

44   cellular localization patterns.

45

Introduction

Proprioception, often referred to as our "sixth sense", is a largely unconscious sensation that allows for the detection of one's own body position and movement in space (1, 2). Proprioceptive signaling is initiated by a subclass of peripheral mechanosensory neurons, called proprioceptors, whose cell bodies reside in the dorsal root ganglia (DRG) or mesencephalic trigeminal nucleus (1, 3, 4). The peripheral axons of proprioceptors innervate skeletal muscle and form mechanosensitive end organs, referred to as muscle spindles and Golgi tendon organs, which are activated by changes is muscle length or force, respectively (1). In proprioceptors, the mechanosensitive ion channel Piezo2 transduces changes in muscle movement into electrical signals that give rise to sustained trains of action potentials, which are subsequently transmitted to spinal cord circuits (4, 5). Indeed, patients harboring Piezo2 loss-of-function mutations have impaired proprioception in the absence of visual input (6). Recently, we determined that the voltage-gated sodium channel (Na_V), $Na_V1.1$, is also essential for mammalian proprioception, and plays a specific role in maintaining proprioceptor firing during sustained muscle stretch (7). Furthermore, we determined $Na_V1.1$ to be haploinsufficient for proprioceptor function and motor behaviors, which is consistent with the clinical manifestations associated with the thousands of human disease-causing mutations associated with its gene, *Scn1a*. Surprisingly, $Na_V1.1$ was not required for muscle proprioceptor responses to dynamic muscle movement or vibration. This raises the question as to whether Na_Vs play distinct roles in encoding proprioceptive signals.

In addition to $Na_V1.1$, proprioceptors also express $Na_V1.6$ and $Na_V1.7$ (7). $Na_V1.7$ is most notable for its role in pain signaling, whereby gain- or loss-of-function mutations in *Scn9a*, the gene encoding $Na_V1.7$, cause congenital hypersensitivity or insensitivity to pain, respectively (8, 9) . Mice and humans lacking $Na_V1.7$, however, do not have reported motor deficits, indicating a limited role for this channel in proprioception at the behavioral level (9, 10). Conversely, the gene

71 encoding $Na_V1.6$, *Scn8a*, is linked to various pathophysiological conditions associated with motor

72 impairments, such as developmental epileptic encephalopathy and ataxia (*11*). Furthermore, global

73 inactivation of *Scn8a* in mice leads to hind limb paralysis and death by postnatal day (P) 21 (*12*).

74 In cerebellar Purkinje neurons, loss of $Na_V1.6$ significantly reduces spontaneous activity and leads

75 to impairments in motor coordination (*13*). While these data highlight critical roles for $Na_V1.6$ in

76 brain-mediated motor control, $Na_V1.6$ function remains understudied in the peripheral nervous

77 system, and how this channel contributes to proprioception is unknown. Importantly, understanding

78 the unique contributions of Na_Vs to peripheral proprioception will enhance our mechanistic

79 understanding of the sensorimotor phenotypes associated with various Na_V channelopathies.

80

81 In the present study, we set out to determine whether Na_Vs plays distinct or redundant roles in

82 proprioceptive signaling, focusing on the contributions of $Na_V1.1$ and $Na_V1.6$. The use of a *Pvalb-*

83 *Cre* mouse line to drive Na_V deletion in proprioceptors is not feasible due to parvalbumin expression

84 in the brain and spinal cord (*7, 14, 15*). Thus, we used a somatosensory-neuron wide genetic

85 targeting strategy to conditionally deleted $Na_V1.6$ (*Pirt^{Cre/+};Scn8a^{fl/fl}*, $Na_V1.6^{cKO}$) and found this

86 resulted in severe impairments in motor coordination that were phenotypically distinct from those

87 we previously observed in mice lacking $Na_V1.1$ in somatosensory neurons (*Pirt^{Cre/+};Scn1a^{fl/fl}*,

88 $Na_V1.1^{cKO}$, *7*). In line with behavioral observations, *ex vivo* proprioceptor muscle-nerve recordings

89 showed neurotransmission in response to both dynamic and static muscle movement was abolished

90 in the absence of $Na_V1.6$, which contrasts with our prior finding of a selective role for $Na_V1.1$ in

91 proprioceptor encoding of static muscle stretch. Electrophysiological recordings of the

92 proprioceptor-mediated monosynaptic reflex in the spinal cord further confirmed an essential, albeit

93 developmentally dependent, role for $Na_V1.6$ in proprioceptor synaptic function, whereas $Na_V1.1$

94 was found to be dispensable. $Na_V1.6^{cKO}$ mice also exhibited abnormal muscle spindle end organ

95 structure, which was not observed in $Na_V1.1^{cKO}$ mice, suggesting severely but not moderately

96   impaired proprioceptive signaling interferes with proprioceptor end organ development.

97   Surprisingly, we also observed non-cell-autonomous deficits in skeletal muscle development in

98    $Na_V1.6^{cKO}$ mice, but not $Na_V1.1^{cKO}$ mice, that are suggestive of blocked hypertrophy during

99   development. Finally, cellular localization experiments found $Na_V1.1$ and $Na_V1.6$ occupy discrete

100   excitable domains in proprioceptor muscle spindles, which we predict underlies their unique roles

101   in electrical transmission.

102

103   Collectively, our findings reveal that $Na_V1.1$ and $Na_V1.6$ are both essential to proprioceptive

104   signaling but have independent and non-redundant functions. The differential contribution of

105    $Na_V1.1$ and $Na_V1.6$ to the activity of individual somatosensory neuron subtypes has not been

106   investigated, and we hypothesize our findings are broadly applicable to other somatosensory

107   neurons that rely on these channels for neuronal signaling.

108

Results
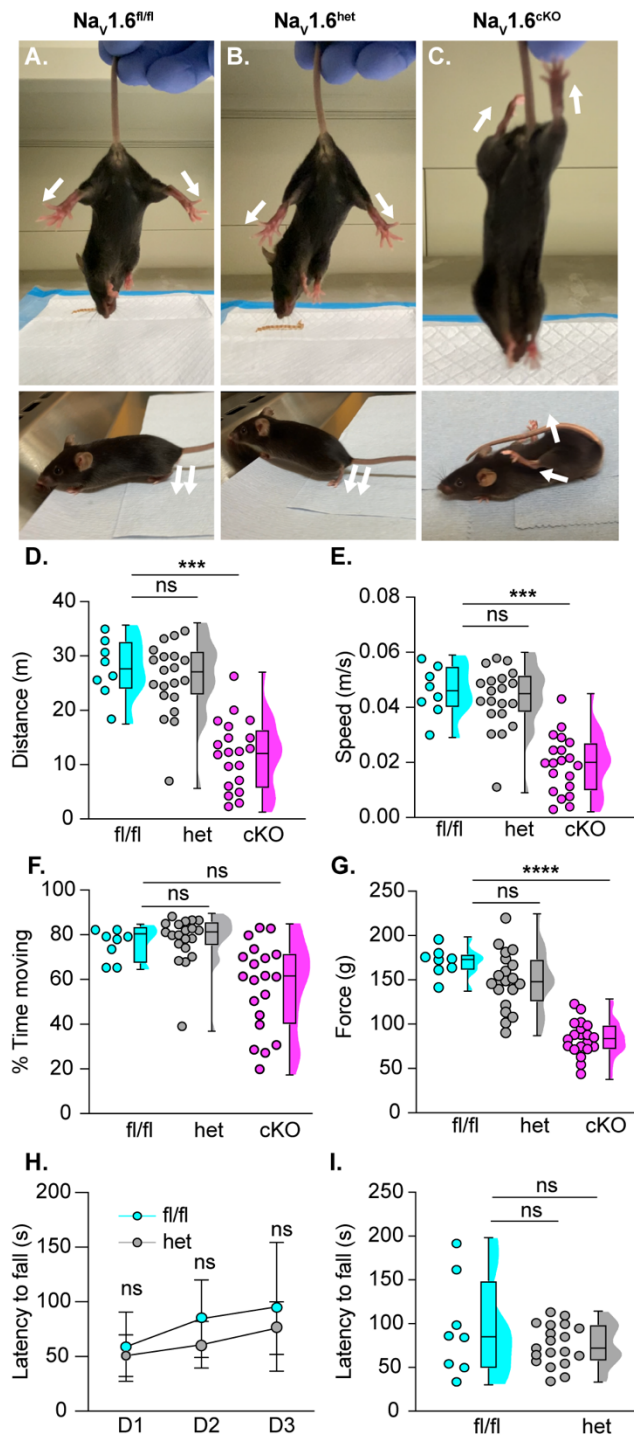
Fig. 1. Na$_V$1.6 is required for somatosensory neuron-driven motor behaviors and function. Representative images showing limb position of adult Na$_V$1.6$^{fl/fl}$ (**A**), Na$_V$1.6^{het} (**B**), and Na$_V$1.6^{cKO} (**C**) mice suspended from the tail (above) and on flat surface (below). White arrows indicate the direction of hind limbs. Quantification of distance traveled (**D,** Na$_V$1.6^{het} p > 0.999 , Na$_V$1.6^{cKO} p = 0.0003, compared to Na$_V$1.6$^{fl/fl}$), average speed (**E,** Na$_V$1.6^{het} p > 0.999 , Na$_V$1.6^{cKO} p = 0.0003), and the percent of time spent moving (**F,** Na$_V$1.6^{het} p > 0.999, Na$_V$1.6^{cKO} p = 0.05, compared to Na$_V$1.6$^{fl/fl}$) for Na$_V$1.6$^{fl/fl}$ (cyan), Na$_V$1.6^{het} (grey), and Na$_V$1.6^{cKO} (magenta) mice as measured in the open field assay for a 10-minute testing period. (**G**) Average grip force in grams measured

119 across 6 consecutive trials; $Na_V1.6^{het}$ p = 0.4947, $Na_V1.6^{cKO}$ p < 0.0001, compared to $Na_V1.6^{fl/fl}$.
120 (**H**) Average latency to fall from the rotarod across three consecutive training days. No statistically
121 significant genotype-dependent difference was observed (p = 0.1342). (**I**) Average latency to fall
122 on third day of testing ($Na_V1.6^{het}$ p = 0.3943, compared to $Na_V1.6^{fl/fl}$). Each dot represents one
123 animal, except in (**H**) were each dot represents the mean across animals. Box and whisker plots
124 represent maximum, minimum, median, upper and lower quartiles of data sets. A Kruskal-Wallis
125 test with Dunn's multiple comparisons (**D** to **G**), a Two-way ANOVA with Sidak's multiple
126 comparisons (**H**), and a Welch's T-test (**I**) were used to determine statistical significance. $Na_V1.6^{fl/fl}$
127 N = 8, $Na_V1.6^{het}$ N=20, $Na_V1.6^{cKO}$ N=20.

128

129 **Genetic ablation of $Na_V1.6$ in sensory neurons leads to profound motor coordination deficits.**

130 To examine the *in vivo* role of $Na_V1.6$ in sensory-driven motor behaviors, we generated a mouse

131 line in which $Na_V1.6$ is deleted in all peripheral sensory neurons: *$Pirt^{Cre/+}Scn8a^{fl/fl}$* (hereafter

132 referred to as $Na_V1.6^{cKO}$), an approach we previously used to investigate $Na_V1.1$ function in

133 proprioception (*7*). While not selective to proprioceptors, this approach avoids significant off-target

134 effects on the central nervous system, which include premature death and seizures (*14*). $Na_V1.6^{cKO}$

135 mice displayed extreme motor deficits that were absent in mice retaining a single copy ($Na_V1.6^{het}$)

136 or both copies ($Na_V1.6^{fl/fl}$) of *Scn8a* (Fig. 1). Motor deficits included abnormal hind limb position

137 when suspended by the tail (Fig. 1 A to C, top; movie S1) or when placed on a flat surface (Fig. 1

138 A to C, bottom; movie S2) and an inability to use the tail to guide movements. The motor phenotype

139 produced by $Na_V1.6$ deletion was more severe than the phenotype we observed following deletion

140 of $Na_V1.1$ in sensory neurons (*$Pirt^{Cre/+};Scn1a^{fl/fl}$*, $Na_V1.1^{cKO}$, *7*). Interestingly, however, $Na_V1.6^{cKO}$

141 mice did not display the tremor-like movements we previously observed in $Na_V1.1^{cKO}$ animals,

142 highlighting a behaviorally distinct phenotype between the two models. We quantified spontaneous

143 locomotion in the open-field and found that $Na_V1.6^{cKO}$ animals traveled significantly less distance

144 (Fig. 1D) and were slower (Fig. 1E) compared to $Na_V1.6^{het}$ and $Na_V1.6^{fl/fl}$ animals. There were no

145 genotype dependent differences in time spent moving (Fig. 1F), suggesting that motivation to move

146 is not impaired in $Na_V1.6^{cKO}$ mice. Time spent in the center was also not different between

147 genotypes (Fig. S1, A). Furthermore, we did not observe any sex-dependent differences between

148 genotypes (Fig. S1 B to D). Using a grip strength meter, we quantified grip force when all four

149   paws were placed on a metal grid and found that $Na_V1.6^{cKO}$ animals had a significantly reduced

150   grip strength compared to other genotypes (Fig. 1G). We next assessed motor coordination using

151   the rotarod; however, the severe motor phenotype of $Na_V1.6^{cKO}$ mice precluded their testing in this

152   assay. We did not observe genotype dependent differences in latency to fall between $Na_V1.6^{het}$ and

153    $Na_V1.6^{fl/fl}$ animals across training days (Fig. 1H) or on the final day of testing (Fig. 1I). Collectively,

154   these data show that genetic ablation of $Na_V1.6$ in sensory neurons leads to severe motor deficits

155   that are distinct to those due to $Na_V1.1$ deletion. Interestingly, we previously reported that $Na_V1.1$

156   was haploinsufficient in sensory neurons for motor behaviors; however, these results suggest a

157   single copy of $Na_V1.6$ is sufficient to drive normal motor function at the behavioral level.

158   Nevertheless, we did observe $Na_V1.6$ haploinsufficiency at the afferent level.
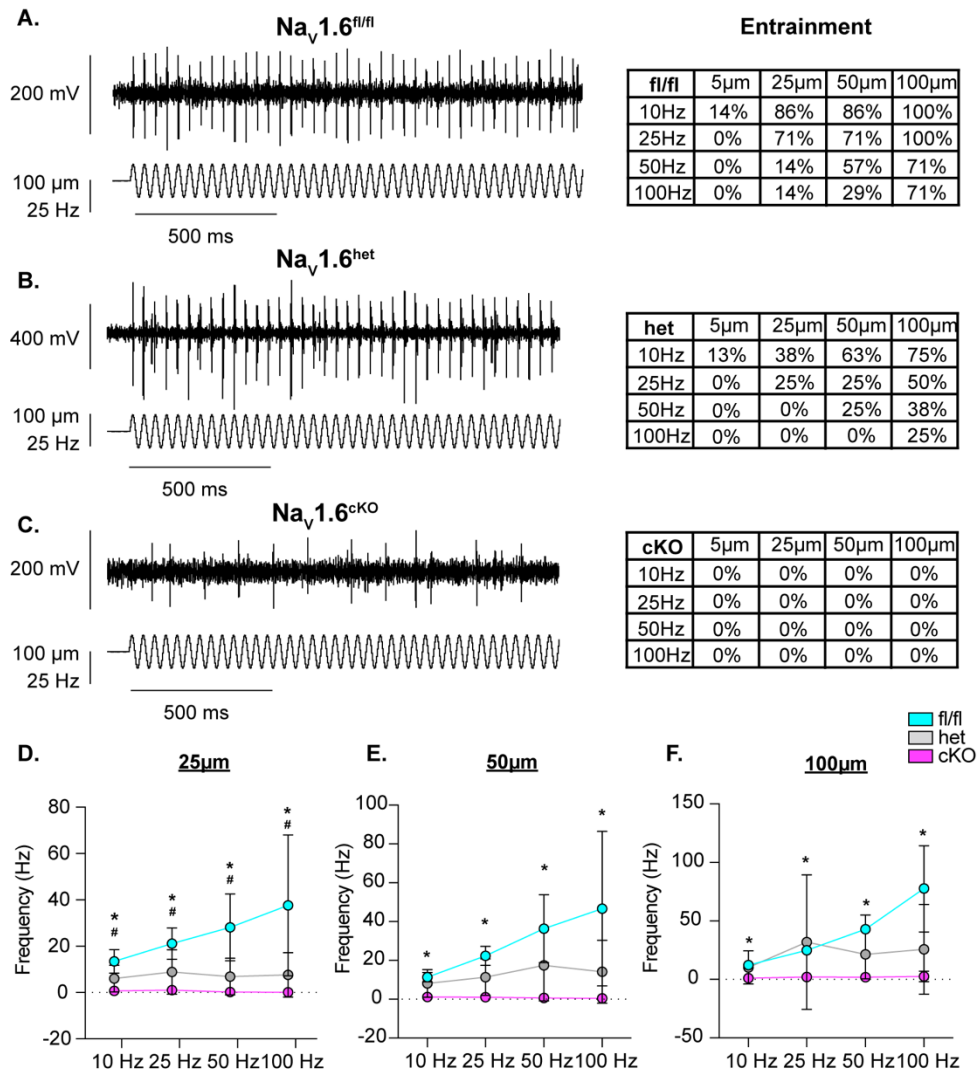
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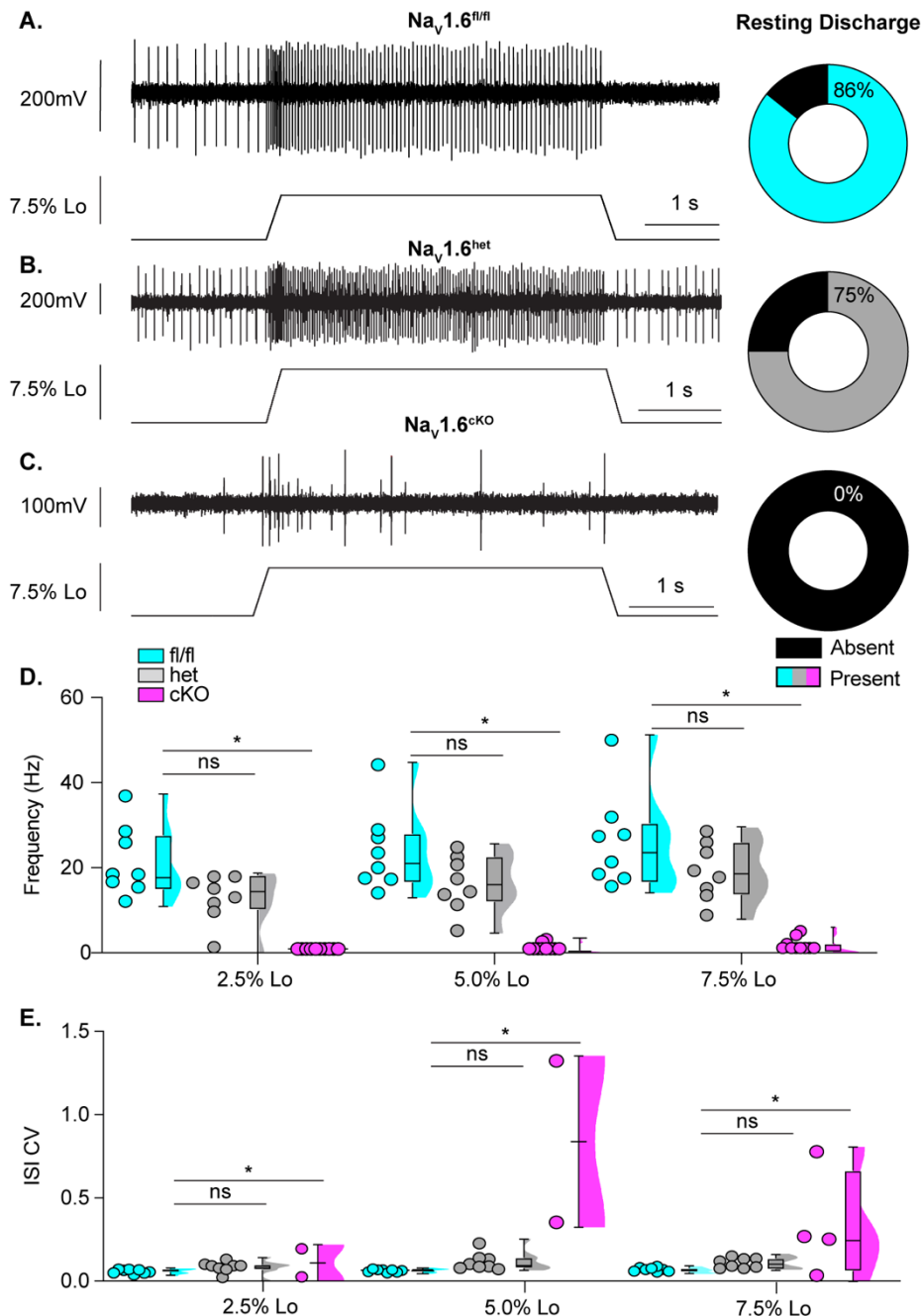
160 **Fig. 2. Loss of Na$_V$1.6 abolishes muscle proprioceptor static stretch sensitivity.** Representative
161 responses to ramp-and-hold muscle stretch at 7.5% of optimal length (Lo) in Na$_V$1.6$^{fl/fl}$ (**A**),
162 Na$_V$1.6^{het} (**B**), and Na$_V$1.6^{cKO} (**C**) muscle proprioceptors. The percentage of afferents that displayed
163 resting discharge at Lo are represented by the pie charts to the right (black indicates absence of
164 resting discharge). (**D**) Quantification of afferent firing frequency 3.25 to 3.75 seconds into stretch
165 protocol. Na$_V$1.6$^{fl/fl}$ (cyan), Na$_V$1.6^{het} (grey), and Na$_V$1.6^{cKO} (magenta). Na$_V$1.6^{het} p = 0.178,
166 Na$_V$1.6^{cKO} p = 0.001, compared to Na$_V$1.6$^{fl/fl}$. (**E**) Firing regularity was quantified as the coefficient
167 of variation of the interspike interval (ISI CV) 1.5 to 3.5 seconds into the stretch protocol. Na$_V$1.6^{het}
168 p = 0.669, Na$_V$1.6^{cKO} p = 0.000, compared to Na$_V$1.6$^{fl/fl}$. In 6 out of 10 animals we observed no
169 response to stretch and therefore could only include the quantifiable responses from 4 afferents
170 from Na$_V$1.6^{cKO} mice. Only quantifiable responses were included in statistical analyses in **D** and **E**.
171 Box and whisker plots represent maximum, minimum, median, upper and lower quartiles of data

172 sets. Each dot represents a single afferent. A two-way mixed-design ANOVA (Dunnett's post-hoc
173 comparison) was used to determine statistical significance in **D** and **E**. $Na_V1.6^{fl/fl}$ n = 8, N=7;
174 $Na_V1.6^{het}$ n=8, N=8; $Na_V1.6^{cKO}$ n=4, N=10. n = afferents, N = mice.

$Na_V1.6$ is required for transmission of proprioceptive signals from muscle spindle afferents.

177 Our prior work found that $Na_V1.1$ plays a key role maintaining muscle afferent activity only during

178 static muscle stretch (*7*). Deletion of $Na_V1.1$ had no effect on muscle afferent responses to dynamic

179 muscle movement or vibratory stimuli. To test whether $Na_V1.6$ serves a similarly specific role in

180 proprioceptive transmission, we used an *ex vivo* muscle nerve preparation to investigate

181 proprioceptor activity from muscle spindle afferents (*16*). First, we tested afferent firing in response

182 to a series of ramp and hold stretches (Fig. 2). Afferents from $Na_V1.6^{fl/fl}$ mice displayed consistent

183 firing throughout the duration of a 4 s ramp and hold stretch protocol and had a high likelihood of

184 resting discharge (Fig. 2A), consistent with wild-type Group Ia and II proprioceptor responses.

185 Afferents from $Na_V1.6^{het}$ mice had a similar prevalence of resting discharge compared to $Na_V1.6^{fl/fl}$

186 mice and did not exhibit any significant differences in firing during ramp and hold stretches (Fig.

187 2B). Strikingly, afferents from $Na_V1.6^{cKO}$ mice never possessed resting discharge and

188 neurotransmission during ramp and hold stretches were nearly abolished (Fig. 2C). In 6 out of the

189 10 mice tested, no stretch-sensitive electrical activity was observed despite the muscle exhibiting

190 healthy twitch contractions. We quantified afferent properties by examining instantaneous firing

191 frequencies and found a significant reduction in firing in $Na_V1.6^{cKO}$ afferents compared to $Na_V1.6^{fl/fl}$

192 afferents across all stretch lengths (Fig. 2D). There were no significant genotype dependent

193 differences in firing between $Na_V1.6^{het}$ and $Na_V1.6^{fl/fl}$ afferents. We also quantified the regularity

194 of afferent firing by measuring the coefficient of variation of the interspike interval (ISI CV). ISI

195 CV was similar between $Na_V1.6^{fl/fl}$ and $Na_V1.6^{het}$ afferents but was significantly higher in $Na_V1.6^{cKO}$

196 afferents (Fig. 2E). Together these findings provide strong evidence that $Na_V1.6$ is required for

197 proprioceptor encoding of static stretch.

198

Entrainment

fl/fl	5µm	25µm	50µm	100µm
10Hz	14%	86%	86%	100%
25Hz	0%	71%	71%	100%
50Hz	0%	14%	57%	71%
100Hz	0%	14%	29%	71%

het	5µm	25µm	50µm	100µm
10Hz	13%	38%	63%	75%
25Hz	0%	25%	25%	50%
50Hz	0%	0%	25%	38%
100Hz	0%	0%	0%	25%

cKO	5µm	25µm	50µm	100µm
10Hz	0%	0%	0%	0%
25Hz	0%	0%	0%	0%
50Hz	0%	0%	0%	0%
100Hz	0%	0%	0%	0%

Fig 3. $Na_V1.6$ is required for proprioceptor responses to vibration. Representative traces from $Na_V1.6^{fl/fl}$ (**A**), $Na_V1.6^{het}$ (**B**), and $Na_V1.6^{cKO}$ (**C**) afferents that were able to entrain to a 25 Hz, 100 µm amplitude vibration stimulus. Tables to the right indicate the percentage of afferents that were able to entrain across stimulus frequencies and amplitudes ($Na_V1.6^{fl/fl}$, top; $Na_V1.6^{het}$, middle; $Na_V1.6^{cKO}$, bottom; **D** to **E**) Quantification of firing frequency across vibration amplitudes. At 25µm (**D**) $Na_V1.6^{het}$ p = 0.005 (# denotes significance in $Na_V1.6^{het}$), $Na_V1.6^{cKO}$ p = 0.001, compared to $Na_V1.6^{fl/fl}$ (* denotes significance in $Na_V1.6^{cKO}$). At 50µm (**E**) $Na_V1.6^{het}$ p = 0.053, $Na_V1.6^{cKO}$ p = 0.002, compared to $Na_V1.6^{fl/fl}$. At 100µm (**F**) $Na_V1.6^{het}$ p = 0.414, $Na_V1.6^{cKO}$ p = 0.018 compared to $Na_V1.6^{fl/fl}$. $Na_V1.6^{fl/fl}$ (cyan), $Na_V1.6^{het}$ (grey), and $Na_V1.6^{cKO}$ (magenta). A two-way mixed-design ANOVA (Dunnett's post-hoc comparison) was used to determine statistical in **D** to **F**. Box and whisker plots represent maximum, minimum, median, upper and lower quartiles of data sets. Each dot represents the average afferent response per genotype. $Na_V1.6^{fl/fl}$ n = 8, N=7; $Na_V1.6^{het}$ n=8, N=8; and $Na_V1.6^{cKO}$ n= 4, N=10. n=afferents, N=mice.

Given that $Na_V1.1$ only contributes to proprioceptor afferent firing in response to static muscle stretch, we next asked whether this was also true for $Na_V1.6$. Afferents were tested using a series of sinusoidal vibration protocols at varying frequencies and stimulus amplitudes. In line with the

216 absence of electrical activity during static stretch, $Na_V1.6$ afferents were completely unable to

217 entrain to vibratory stimuli regardless of stimulus amplitude and frequency (Fig. 3). Interestingly,

218 logistic regression analyses of entrainment probability found that compared to $Na_V1.6^{fl/fl}$ afferents,

219 afferents from $Na_V1.6^{het}$ animals were significantly less likely to entrain sinusoidal waves ($p<0.001$;

220 Fig. 3 A and B). This analysis could not be used to assess entrainment probability in $Na_V1.6^{cKO}$

221 afferents because these afferents never entrained to vibration. Consistent with logistic regression

222 analyses, quantification of the instantaneous firing frequency at 25μm amplitude vibrations found

223 significant impairments in the ability of $Na_V1.6^{het}$ afferents to respond to vibration, consistent with

224 the notion that $Na_V1.6$ is partially haploinsufficient at the proprioceptor afferent level (Fig. 3D).

225 Thus, unlike $Na_V1.1$ which only serves a role in maintaining proprioceptor responses to static

226 stretch, we find $Na_V1.6$ plays a direct role in transmitting both dynamic and static muscle

227 movement.

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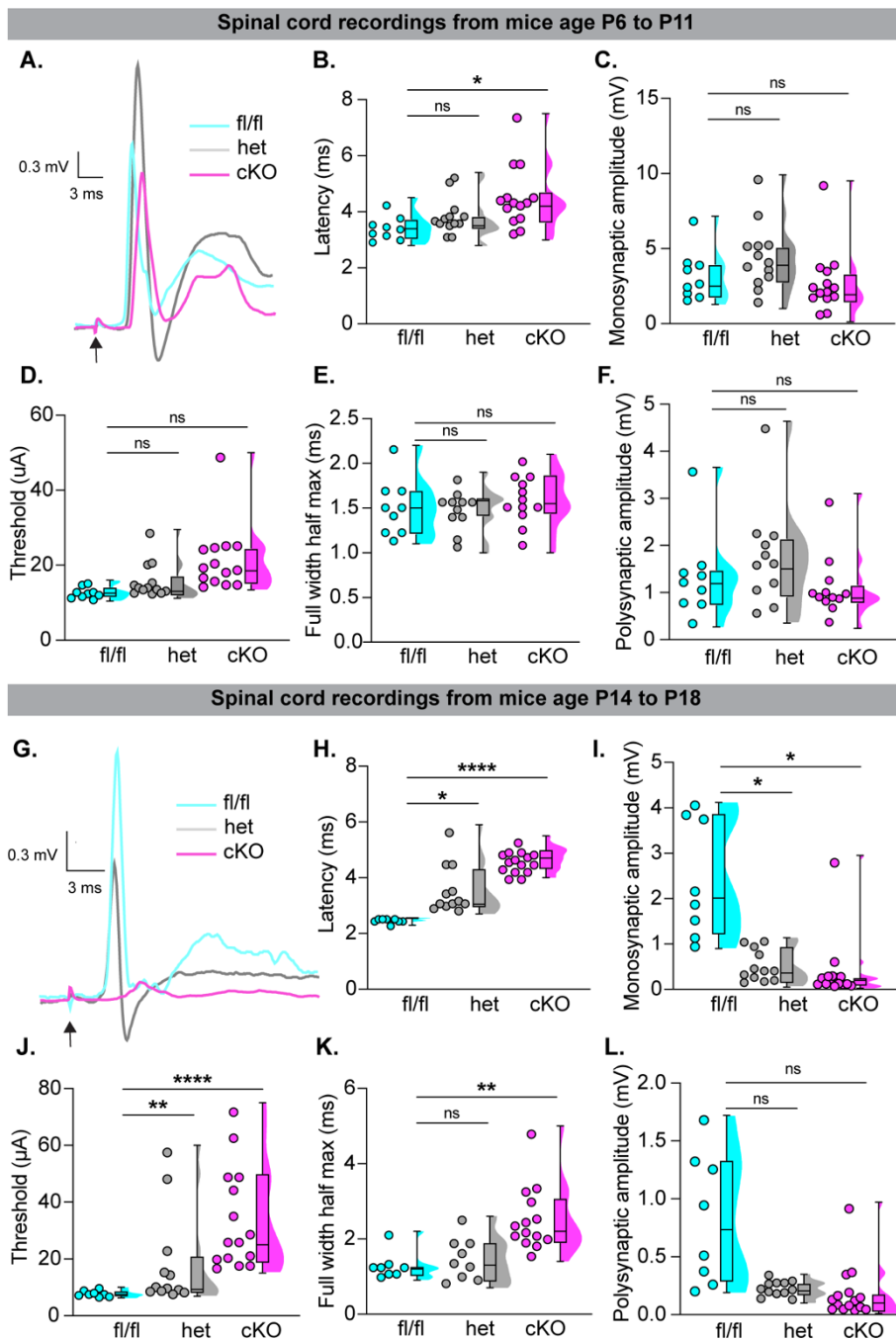
Fig. 4. Na$_V$1.6 plays a developmentally dependent role in proprioceptor synaptic transmission in the spinal cord. (A) Representative monosynaptic reflex responses from Na$_V$1.6$^{fl/fl}$ (cyan), Na$_V$1.6^{het} (grey), and Na$_V$1.6^{cKO} (magenta) hemicords during postnatal days 6 to 11. Quantification of response properties. (B) Response latency, Na$_V$1.6^{het} p = 0.760, Na$_V$1.6^{cKO} p = 0.019, compared to Na$_V$1.6$^{fl/fl}$. (C) Monosynaptic response amplitude, Na$_V$1.6^{het} p = 0.238 , Na$_V$1.6^{cKO} p = 0.640, compared to Na$_V$1.6$^{fl/fl}$. (D) Stimulus threshold, Na$_V$1.6^{het} p = 0.910, Na$_V$1.6^{cKO} p = 0.271, compared to Na$_V$1.6$^{fl/fl}$. (E) Full width half max, Na$_V$1.6^{het} p = 0.999, Na$_V$1.6^{cKO} p = 0.929, compared to Na$_V$1.6$^{fl/fl}$. (F) Polysynaptic response amplitude, Na$_V$1.6^{het} p = 0.514, Na$_V$1.6^{cKO} p = 0.704, compared to Na$_V$1.6$^{fl/fl}$. (G) Representative monosynaptic reflex responses in Na$_V$1.6$^{fl/fl}$ (cyan), Na$_V$1.6^{het} (grey), and Na$_V$1.6^{cKO} (magenta) hemicords during postnatal days 14 to 18. (H) Response latency, Na$_V$1.6^{het} p = 0.023, Na$_V$1.6^{cKO} p < 0.0001, compared to Na$_V$1.6$^{fl/fl}$. (I) Monosynaptic

240 response amplitude, $Na_V1.6^{het}$ p = 0.037, $Na_V1.6^{cKO}$ p = 0.018, compared to $Na_V1.6^{fl/fl}$. (**J**) Stimulus
241 threshold, $Na_V1.6^{het}$ p = 0.164, $Na_V1.6^{cKO}$ p <0.0001 , compared to $Na_V1.6^{fl/fl}$. (**K**) Full width half
242 max, $Na_V1.6^{het}$ p = 0.784, $Na_V1.6^{cKO}$ p <0.0001 , compared to $Na_V1.6^{fl/fl}$. (**L**) Polysynaptic response
243 amplitude, $Na_V1.6^{het}$ p = 0.143, $Na_V1.6^{cKO}$ p = 0.092, compared to $Na_V1.6^{fl/fl}$. Each dot represents
244 a single hemicord. (**A** to **F**) $Na_V1.6^{fl/fl}$ n=9, $Na_V1.6^{het}$ n=13, and $Na_V1.6^{cKO}$ n=14. (**G** to **L**) $Na_V1.6^{fl/fl}$
245 n=8, $Na_V1.6^{het}$ n=12, and $Na_V1.6^{cKO}$ n=15. N=8-15. n=hemicords, N=mice. Box and whisker plots
246 represent maximum, minimum, median, upper and lower quartiles of data sets. A two-way mixed-
247 design ANOVA (Tukey's post-hoc comparison) was used to determine statistical significance.
248
249 **$Na_V1.6$ is essential for proprioceptor synaptic transmission in a developmentally dependent**

250 **manner.**

251 Electrical signals initiated at proprioceptive end organs in skeletal muscle are transmitted to central

252 circuits in the spinal cord. Specifically, proprioceptor Ia afferents directly synapse with alpha motor

253 neurons, comprising the monosynaptic reflex response (*17, 18*). This spinal circuit provides a

254 tractable model to assess proprioceptor synaptic transmission. Our current results demonstrate that

255 $Na_V1.6$ plays a central role in sensory transmission from muscle spindles; thus, we next asked

256 whether the peripheral deficits we observed in *ex vivo* muscle nerve recordings are also evident in

257 proprioceptive circuits in the spinal cord. We used an *ex vivo* hemisected spinal cord preparation

258 and measured properties of the monosynaptic reflex circuit in $Na_V1.6^{fl/fl}$, $Na_V1.6^{het}$, and $Na_V1.6^{cKO}$

259 mice (Fig. 4). We first analyzed responses from mice in early postnatal development (P6 to P11) as

260 all prior work has used this age range for monosynaptic reflex analysis, largely due to technical

261 challenges associated with increased myelination in the ventral horn as development proceeds (*19*).

262 In stark contrast to our muscle-nerve recordings, monosynaptic responses were similar between

263 genotypes at this timepoint (Fig. 4). We only observed a significant difference in response latency

264 in $Na_V1.6^{cKO}$ hemicords compared to $Na_V1.6^{het}$ and $Na_V1.6^{fl/fl}$ hemicords (Fig. 4B). No other

265 genotype dependent differences were observed across quantified parameters (Fig. 4 C to F). These

266 findings suggest that during early postnatal development, $Na_V1.6$ is dispensable for proprioceptor
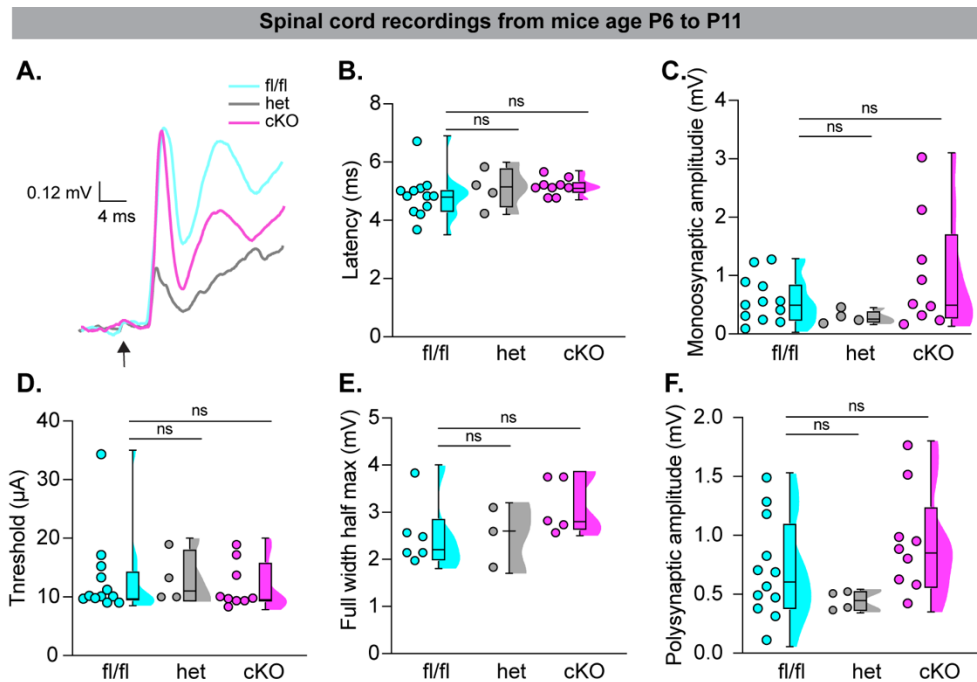
267 synaptic transmission.

268

269   Interestingly, previous studies indicate that proprioceptors are not transcriptionally mature until

270   walking behaviors begin to emerge (*20*), which occurs around P13. Thus, we decided to test

271   monosynaptic responses beyond this time point (P14 to P18). To our knowledge, this is first

272   systematic analysis of the monosynaptic reflex this late in postnatal development. Strikingly, at this

273   age, proprioceptive synaptic transmission is nearly lost in $Na_V1.6^{cKO}$ hemicords (Fig. 4G). We

274   found highly significant genotype dependent differences between $Na_V1.6^{cKO}$ and $Na_V1.6^{fl/fl}$

275   hemicords across all quantified parameters (Fig. 4 H to L). This suggests that following the onset

276   of walking behaviors, $Na_V1.6$ is essential for proprioceptor synaptic transmission onto alpha motor

277   neurons, which is also consistent data from *ex vivo* muscle nerve recordings in adult afferents (Figs.

278   2 and 3). Additionally, we also found significantly increased monosynaptic reflex response

279   latencies and thresholds (Fig. 4H and J), as well as significantly reduced response amplitudes in

280     $Na_V1.6^{het}$ hemicords compared to $Na_V1.6^{fl/fl}$ controls (Fig. 4 I). Finally, when looking within

281   genotypes, we found that unlike in $Na_V1.6^{fl/fl}$ hemicords, which only showed a significant increase

282   in response latency, there was a significant degradation of the monosynaptic reflex response in

283     $Na_V1.6^{het}$ and $Na_V1.6^{cKO}$ hemicords (Table S1). Thus, during postnatal development, $Na_V1.6^{fl/fl}$

284   animals exhibit an enhancement in central proprioceptive signaling, whereas in both $Na_V1.6^{het}$ and

285     $Na_V1.6^{cKO}$ mice, central proprioceptive signaling degrades. This provides additional evidence that

286   at the circuit level, $Na_V1.6$ is haploinsufficient for proprioceptor synaptic function.

287
288   The developmental dependence of the proprioceptor mediated monosynaptic reflex response on

289     $Na_V1.6$ prompted us to investigate motor behaviors in this line at P7 and P14, before and after the

290   onset of weight bearing locomotion, respectively (Fig. S2). We analyzed P7 mice in a righting

291   reflex assay, a hindlimb suspension test, a grasping reflex assay, and quantified hindlimb angle. In

292   line with spinal cord electrophysiology data, behavioral testing at P7 found a minimal role of

293     $Na_V1.6$ across behavioral assays (Fig. S2, A to H); we only observed a significant difference in

294   hindlimb angle at this age. Conversely, when we analyzed motor abilities in a limb coordination

assay at P14, we observed significant differences in functional grasping in both $Na_V1.6^{het}$ and

$Na_V1.6^{cKO}$ mice compared to $Na_V1.6^{fl/fl}$ controls (Fig. S2, I to J). These data highlight a

developmentally-specific contribution of $Na_V1.6$ to proprioceptive synaptic transmission in the

spinal cord, which also manifests at the behavioral level.

Fig. 5. $Na_V1.1$ does not contribute to proprioceptor synaptic transmission prior to the onset of walking behaviors. (**A**) Representative monosynaptic reflex responses from $Na_V1.1^{fl/fl}$ (cyan), $Na_V1.1^{het}$ (grey), and $Na_V1.1^{cKO}$ (magenta) hemicords recorded during postnatal days 6 to 11. (**B** to **D**) Quantification of monosynaptic response properties. (**B**) Response latency, $Na_V1.1^{het}$ p = 0.723, $Na_V1.1^{cKO}$ p = 0.238, compared to $Na_V1.1^{fl/fl}$. (**C**) Monosynaptic response amplitude, $Na_V1.1^{het}$ p = 0.378, $Na_V1.1^{cKO}$ p > 0.999, compared to $Na_V1.1^{fl/fl}$. (**D**) Stimulus threshold, $Na_V1.1^{het}$ p>0.999 , $Na_V1.1^{cKO}$ p>0.999 , compared to $Na_V1.1^{fl/fl}$. (**E**) Full width half max, $Na_V1.1^{het}$ p>0.999 , $Na_V1.1^{cKO}$ p = 0.255, compared to $Na_V1.1^{fl/fl}$. (**F**) Polysynaptic response amplitude, $Na_V1.1^{het}$ p = 0.574, $Na_V1.1^{cKO}$ p = 0.473, compared to $Na_V1.1^{fl/fl}$. Each dot represents a single hemicord. $Na_V1.1^{fl/fl}$ n=12, $Na_V1.1^{het}$ n=4, and $Na_V1.1^{cKO}$ n=9. N=4-10 mice. Box and whisker plots represent maximum, minimum, median, upper and lower quartiles of data sets. A Kruskal-Wallis test with Dunn's multiple comparisons was used to determine statistical significance.

Because we did not observe a role for $Na_V1.6$ in the proprioceptor mediated monosynaptic reflex

response between P6 and P11, we next asked whether instead $Na_V1.1$ was required for

proprioceptor synaptic transmission at this developmental stage. In line with our findings across

$Na_V1.6$ genotypes, monosynaptic reflex responses were genotype-independent in the $Na_V1.1$ mouse

line in this age range (Fig. 5). These data suggest that proprioceptor synaptic transmission at early

318 postnatal development is not dependent on $Na_V1.1$ or $Na_V1.6$ and could indicate Na_V functional

319 redundancy in proprioceptors prior to the onset of walking behaviors. We attempted to measure the

320 monosynaptic reflex in later postnatal development (P14 to P18) in these mice; however, responses

321 across all genotypes were too small to reliably quantify, despite our ability to obtain recordings at

322 this age from mice in the Nav1.6-line (Fig. 4) as well as age matched C57Bl/6J controls (Fig. S3).

323 Thus, we cannot rule out the possibility that $Na_V1.1$ may serve a role in proprioceptor mediated

324 synaptic transmission following the acquisition of weight bearing locomotion. Behavioral testing

325 at P7 did not reveal changes in motor function across genotypes (Fig. S4 A to B). At P14 however,

326 we did observe a significant difference in functional grasping in $Na_V1.1^{cKO}$ mice compared to

327 $Na_V1.1^{fl/fl}$ controls (Fig. S4E), suggesting $Na_V1.1$ is also becomes required for motor coordination

328 at the onset of walking behaviors. These data show that in early postnatal development neither

329 $Na_V1.6$ or $Na_V1.1$ alone is required for proprioceptor synaptic transmission; however, upon the

330 acquisition of weight-bearing locomotion, we find that $Na_V1.6$ becomes functionally dominant at

331 the circuit level, and both $Na_V1.1$ and $Na_V1.6$ are required at the behavioral level.
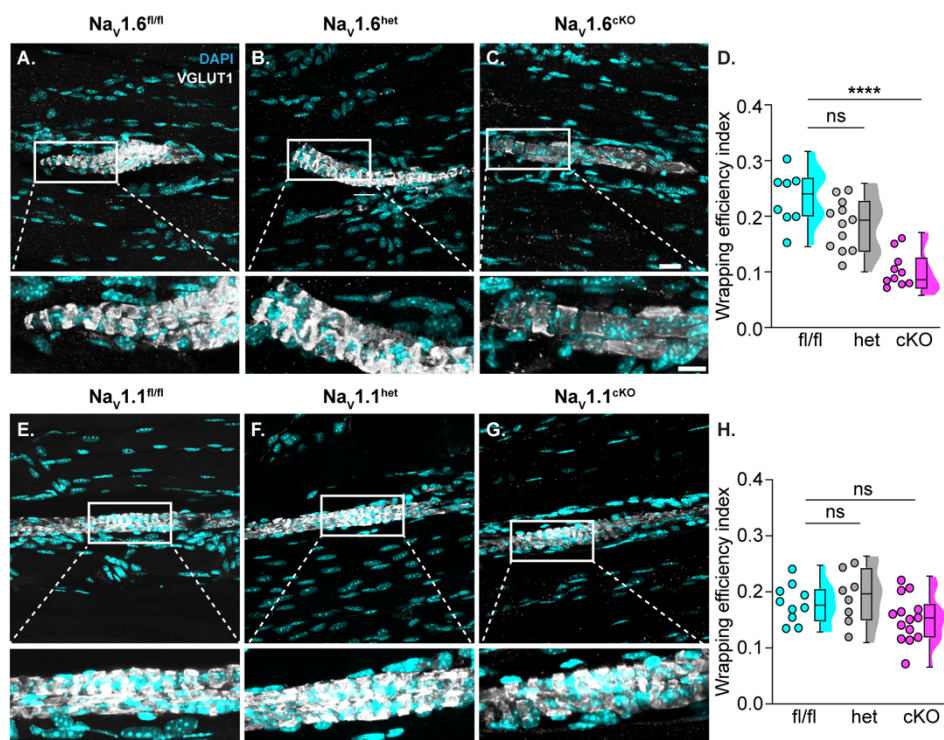
332 **Fig. 6. Electrical signaling deficits associated with $Na_V1.6$- but not $Na_V1.1$-deletion impairs**
333 **muscle spindle development.** Representative confocal images of muscle spindles from (A)

334    $\text{Na}_V1.6^{\text{fl/fl}}$, (**B**) $\text{Na}_V1.6^{\text{het}}$, and (**C**) $\text{Na}_V1.6^{\text{cKO}}$ extensor digitorum longus (EDL) muscle sections (30

335   μm). Images were acquired with a 60x oil 1.4 NA lens. VGLUT1 (grey scale) labels proprioceptor

336   sensory terminals and DAPI (cyan) labels nuclei. Insets below images show the colocalization of

337   sensory terminals with DAPI. (**D**) Quantification of wrapping efficiency index based on

338   colocalization of DAPI with VGLUT1. $\text{Na}_V1.6^{\text{het}}$ p = 0.0658, and $\text{Na}_V1.6^{\text{cKO}}$, p < 0.0001, compared

339   to $\text{Na}_V1.6^{\text{fl/fl}}$. Representative images of muscle spindles from (**E**) $\text{Na}_V1.1^{\text{fl/fl}}$, (**F**) $\text{Na}_V1.1^{\text{het}}$, (**G**)

340    $\text{Na}_V1.1^{\text{cKO}}$. (**H**) Quantification of wrapping efficiency index based on colocalization of DAPI with

341   VGLUT1. $\text{Na}_V1.1^{\text{het}}$, p = 0.762, and $\text{Na}_V1.1^{\text{cKO}}$, p = 0.282, compared to $\text{Na}_V1.1^{\text{fl/fl}}$. Each dot

342   represents a single muscle spindle section. (**D**) $\text{Na}_V1.6^{\text{fl/fl}}$ n=8, $\text{Na}_V1.6^{\text{het}}$ n=12, and $\text{Na}_V1.6^{\text{cKO}}$ n=10.

343   (**H**) $\text{Na}_V1.1^{\text{fl/fl}}$ n=10, $\text{Na}_V1.1^{\text{het}}$ n=8, and $\text{Na}_V1.1^{\text{cKO}}$ n=14. N=3 mice per genotype. Box and whisker

344   plots represent maximum, minimum, median, upper and lower quartiles of data sets. A one-way

345   ANOVA (Dunnett's post-hoc comparison) was used to determine statistical significance. Scale bar

346   = 20 μm. Inset scale bar = 10 μm.

347

348   **Severely, but not moderately, impaired proprioception results in deficits in muscle spindle**

349   **development.**

350   A recent study found that loss of Piezo2 or $\text{Na}_V1.6$ in sensory neurons led to changes in tactile

351   sensory neuron end organ development (*21*). This study raised the possibility that $\text{Na}_V1.6$ may also

352   regulate muscle spindle development. As with our prior examination of $\text{Na}_V1.1^{\text{cKO}}$ mice compared

353   to controls, we did not observe a reduction in the overall number of proprioceptors in DRG sections

354   (identified by *Pvalb* and *Runx3* colocalization) between $\text{Na}_V1.6^{\text{fl/fl}}$, $\text{Na}_V1.6^{\text{het}}$, and $\text{Na}_V1.6^{\text{cKO}}$ mice

355   (Fig. S5 B). We next examined the structure of muscle spindles in $\text{Na}_V1.6^{\text{cKO}}$ mice by performing

356   immunohistochemistry against vesicular glutamate transporter 1 (VGLUT1) to visualize muscle

357   spindle sensory wrappings in sections of extensor digitorum longus muscle. Qualitative observation

358   of muscle spindles from $\text{Na}_V1.6^{\text{cKO}}$ mice show striking structural abnormalities in sensory

359   wrappings that were not present in $\text{Na}_V1.6^{\text{fl/fl}}$ or $\text{Na}_V1.6^{\text{het}}$ animals (Fig. 6 A to C). To validate that

360   the structural changes we observed were not due disruptions in VGLUT1 expression, a subset of

361   experiments were conducted with both VGLUT1 and the pan-neuronal marker βIII-tubulin. Both

362   antibodies showed highly similar levels of immunoreactivity, indicating VGLUT1 is a good proxy

363   for muscle spindle structure (Fig. S6). To our knowledge there is no standardized method to

364   quantitatively assess the structure of muscle spindles. Thus, we devised a quantitative method to

365   examine muscle spindle sensory terminals by measuring the colocalization of VGLUT1$^+$ sensory

366   wrappings around clusters of DAPI-positive nuclei, which represent intrafusal muscle fibers. By

367   normalizing the number of wrappings to muscle spindle length, we calculated a wrapping efficiency

368   index. We found that compared to $Na_V1.6^{fl/fl}$ and $Na_V1.6^{het}$ animals, muscle spindles from

369    $Na_V1.6^{cKO}$ mice had significantly reduced wrapping efficiency indices, demonstrating that $Na_V1.6$

370   in sensory neurons is required for muscle spindle development (Fig. 6D). In our prior work, we

371   qualitatively reported that loss of $Na_V1.1$ does not change muscle spindle structure (*7*). To confirm

372   our previous findings using this quantitative approach, we analyzed the wrapping efficiency index

373   of muscle spindles from $Na_V1.1^{cKO}$ mice compared to $Na_V1.1^{het}$ and $Na_V1.1^{fl/fl}$ controls (Fig. 6 E to

374   H). In line with our previous work, $Na_V1.1$ is not required for muscle spindle development as

375   wrapping efficiency indices were not significantly different between genotypes (Fig. 6H). Thus, the

376   above results show that muscle spindle development is impaired when proprioceptive signaling is

377   severely, but not moderately, disrupted.

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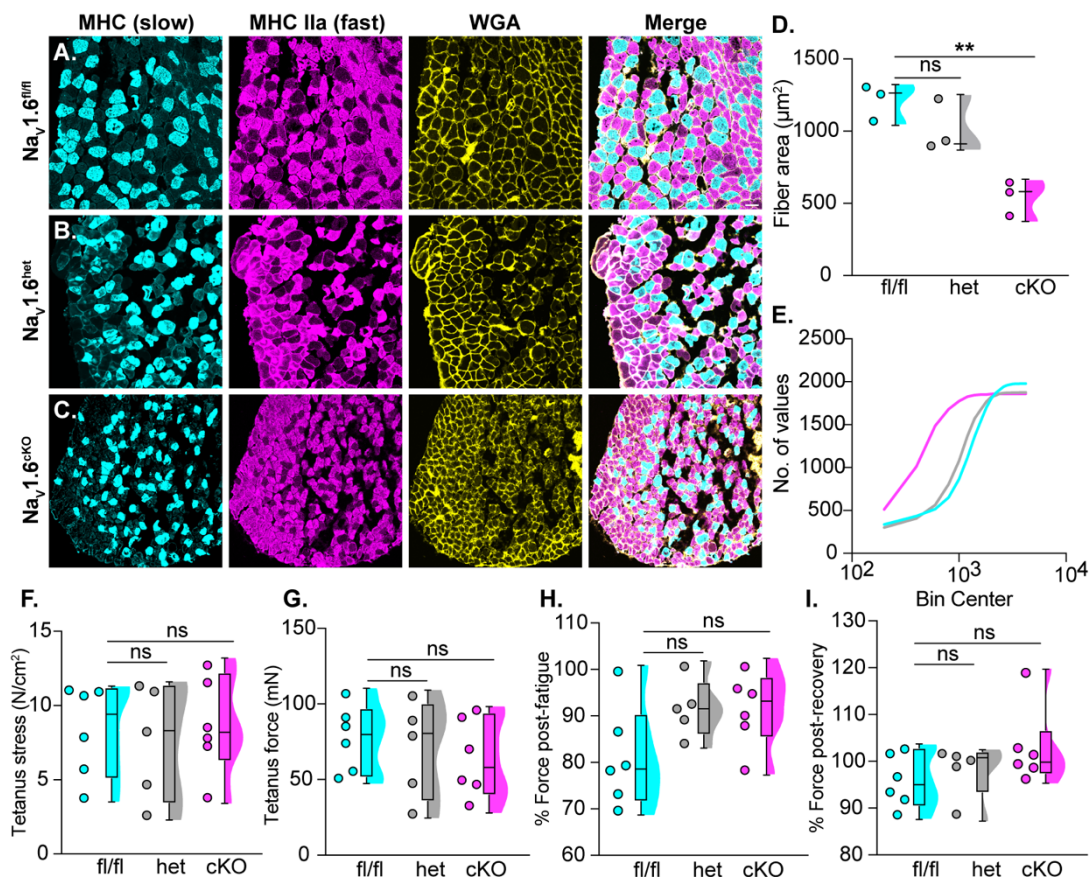
Fig 7. Loss of proprioceptive feedback alters skeletal muscle development in Na$_V$1.6^{cKO} mice.
Representative images of muscle fibers from the soleus of (**A**) Na$_V$1.6$^{fl/fl}$, (**B**) Na$_V$1.6^{het}, and (**C**) Na$_V$1.6^{cKO} mice. Images were acquired with a 20x 0.75 NA air lens. Myosin heavy chain (MHC), labels slow twitch muscle fibers (cyan), MHC type IIa labels fast twitch muscle fibers (magenta), and wheat germ agglutinin (WGA, yellow) labels the cell membrane of muscle fibers. (**D and E**) Quantification of muscle fiber anatomy. (**D**) Fiber area, Na$_V$1.6^{het} p = 0.337, Na$_V$1.6^{cKO} p = 0.006, compared to Na$_V$1.6$^{fl/fl}$. (**E**) cumulative distribution plots showing the muscle fiber area in the soleus between Na$_V$1.6$^{fl/fl}$ (cyan), Na$_V$1.6^{het} (grey), and Na$_V$1.6^{cKO} (magenta) mice. (**F to I**) Quantification of intrinsic properties of soleus muscle. (**F**) Tetanus stress, Na$_V$1.6^{het} p = 0.926, Na$_V$1.6^{cKO} p = 0.990, compared to Na$_V$1.6$^{fl/fl}$. (**G**) Tetanus force, Na$_V$1.6^{het} p = 0.925, Na$_V$1.6^{cKO} p = 0.690, compared to Na$_V$1.6$^{fl/fl}$. (**H**) Percentage of force post-fatigue, Na$_V$1.6^{het} p = 0.189, Na$_V$1.6^{cKO} p = 0.150, compared to Na$_V$1.6$^{fl/fl}$. (**I**) Percentage of force post-recovery, Na$_V$1.6^{het} p = 0.851, Na$_V$1.6^{cKO} p = 0.293, compared to Na$_V$1.6$^{fl/fl}$. Each dot represents a single animal. Box and whisker plots represent maximum, minimum, median, upper and lower quartiles of data sets. A one-way ANOVA (Dunnett's post-hoc comparison) was used to determine statistical significance. Scale bar=50 μm.

Proprioceptive feedback is required for normal skeletal muscle development.

Global inactivation of Na$_V$1.6 leads to severe motor impairments accompanied by atrophy of skeletal muscle (*22*). It was hypothesized that these deficits were caused by loss of signal transmission from motor neurons; however, whether impaired proprioceptive feedback onto motor neurons is sufficient to impair skeletal muscle development has not been directly investigated. To address this, we analyzed skeletal muscle anatomy and function in the Na$_V$1.6 mouse line. We collected soleus muscle from mice and labeled for slow (Type I) and fast (Type IIa) twitch muscle fibers. Muscle fibers from Na$_V$1.6^{cKO} mice had a visible reduction in muscle fiber size compared to fibers from Na$_V$1.6^{het} and Na$_V$1.6$^{fl/fl}$ mice (Fig. 7 A to C). To quantify these changes, we took an unbiased approach and measured muscle fiber properties using a semi-automatic muscle fiber analysis software in MATLAB (*23*). In agreement with qualitative observation, muscle fibers from Na$_V$1.6^{cKO} mice displayed a significant decrease in fiber area compared to Na$_V$1.6^{het} and Na$_V$1.6$^{fl/fl}$ muscle (Fig. 7 D). A cumulative distribution plot shows the spread of muscle fiber area across genotypes (Fig. 7E). The proportion of Type I and Type IIa fibers were also similar between genotypes and were within the expected percentages for soleus muscle in wildtype animals (Fig. S7 A and B, *24, 25*). Furthermore, we found that the size of both Type I and Type IIa fibers in Na$_V$1.6^{cKO} mice were significantly reduced compared to other genotypes, indicating the changes in

412   muscle fiber diameter and area were not fiber-type specific (Fig S7 C and D). We next examined

413   whether the developmental changes in muscle fiber properties corresponded to alterations in

414   intrinsic muscle strength and fatiguability; however, we found no differences in muscle function

415   across genotypes (Fig. 7 F to I). Collectively, these reveal a non-cell autonomous role for

416   proprioceptive feedback in skeletal muscle development.

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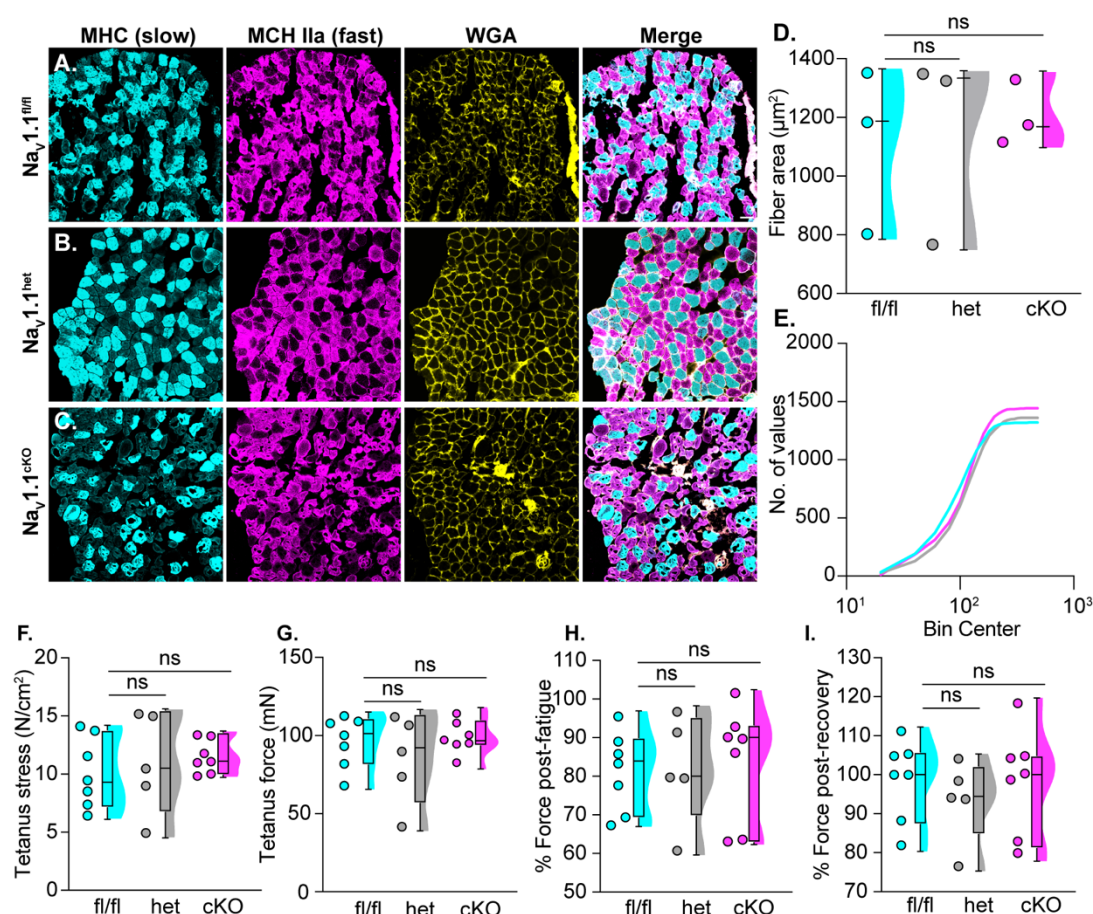
418 **Fig. 8. Mildly impaired proprioceptive feedback does not impair skeletal muscle development.**
419 Images of muscle fibers from (**A**) $Na_V1.1^{fl/fl}$, (**B**) $Na_V1.1^{het}$, and (**C**) $Na_V1.6^{cKO}$ soleus muscle.
420 Images were acquired with a 20x 0.75 NA air lens. Myosin heavy chain (MHC) labels slow twitch
421 muscle fibers (cyan), MHC type IIa labels fast twitch muscle fibers (magenta), and wheat germ
422 agglutinin (WGA, yellow) labels the cell membrane of muscle fibers. (**D** and **E**) Quantification of
423 muscle fiber anatomy. (**D**) Fiber area, $Na_V1.1^{het}$ p = 0.9827, $Na_V1.1^{cKO}$ p = 0.880, compared to
424 $Na_V1.1^{fl/fl}$. (**E**) cumulative distribution plots showing the muscle fiber area between $Na_V1.1^{fl/fl}$
425 (cyan), $Na_V1.1^{het}$ (grey), and $Na_V1.1^{cKO}$ (magenta). (**F** to **I**) Quantification of intrinsic properties of
426 soleus muscle. (**F**) Tetanus stress, $Na_V1.1^{het}$ p = 0.841, $Na_V1.1^{cKO}$ p = 0.596, compared to $Na_V1.1^{fl/fl}$.
427 (**G**) Tetanus force, $Na_V1.1^{het}$ p = 0.624, $Na_V1.1^{cKO}$ p = 0.978, compared to $Na_V1.1^{fl/fl}$. (**H**)
428 Percentage of force post-fatigue, $Na_V1.1^{het}$ p = 0.999, $Na_V1.1^{cKO}$ p = 0.934, compared to $Na_V1.1^{fl/fl}$.
429 (**I**) Percentage of force post-recovery, $Na_V1.1^{het}$ p = 0.724, $Na_V1.1^{cKO}$ p = 0.993, compared to
430 $Na_V1.1^{fl/fl}$. Each dot represents a single animal. Box and whisker plots represent maximum,

431 minimum, median, upper and lower quartiles of data sets. A one-way ANOVA (Dunnett's post-hoc

432 comparison) was used to determine statistical significance. Scale bar=50 μm.

433

434 We next asked whether moderately impaired proprioceptive signaling due to loss of $Na_V1.1$ had the

435 same effect on skeletal muscle development or function. We examined muscle fiber composition

436 of $Na_V1.1$ mice of all genotypes and did not observe any significant changes in fiber area (Fig. 8 A

437 to E). Additionally, the proportion of Type I and Type IIa fibers were similar between genotypes

438 (Fig. S7 E to F). Functional analysis of intrinsic muscle properties in the soleus of $Na_V1.1^{cKO}$ mice

439 did not differ compared to $Na_V1.1^{het}$ or $Na_V1.1^{fl/fl}$ mice (Fig. 8 F to I), suggesting moderately

440 impaired proprioceptive feedback does not result in deficits in skeletal muscle developmental at the

441 anatomical or functional levels. Taken together, these experiments unveil a novel role for

442 proprioceptive feedback in skeletal muscle development.

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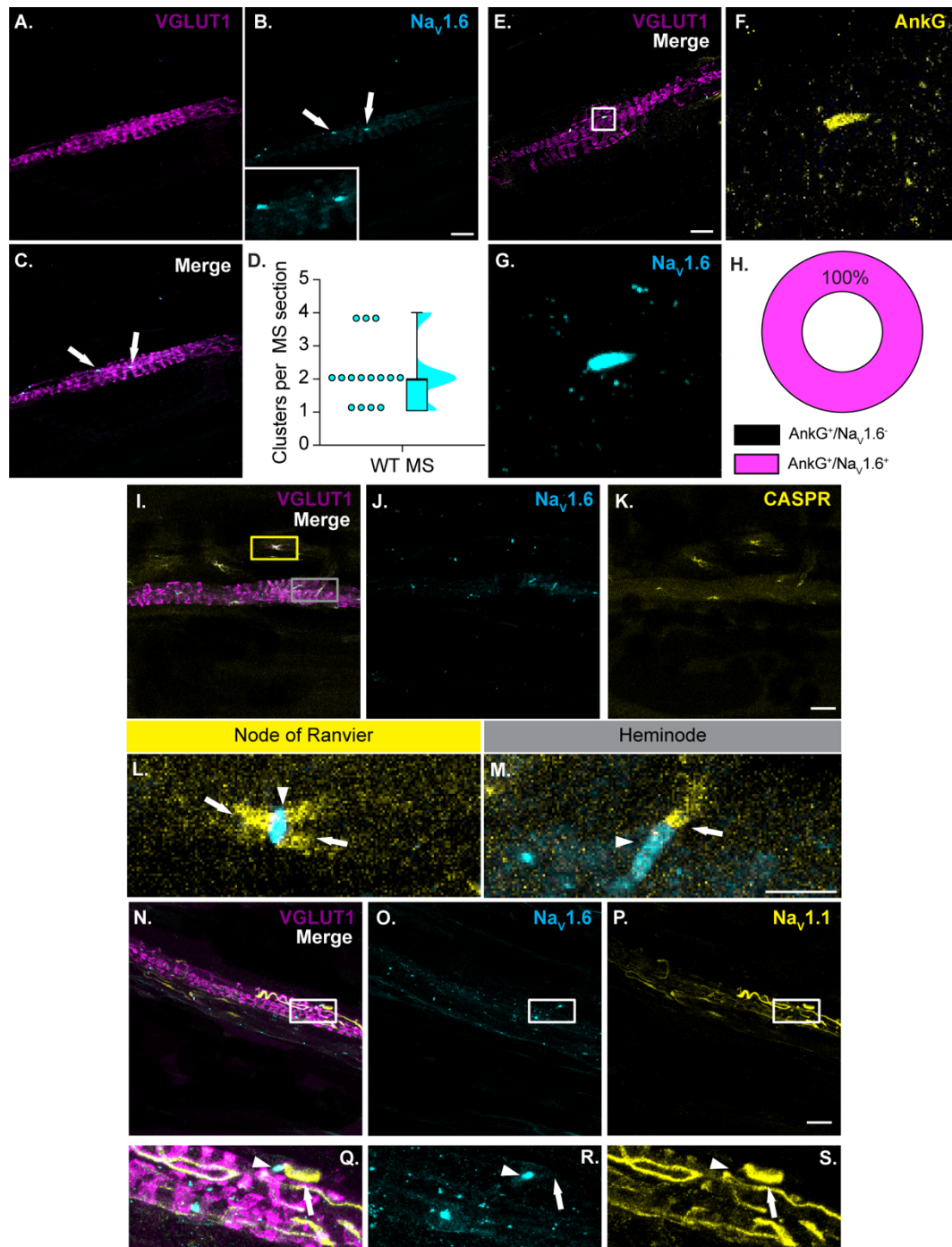
Fig 9. Na$_V$1.1 and Na$_V$1.6 localize to discrete cellular regions in muscle spindles. VGLUT1 (magenta, **A**) labeled muscle spindles express clusters of Na$_V$1.6 (cyan, **B** and **C**). Arrows denote clusters of Na$_V$1.6. (**D**) Quantification of the number of Na$_V$1.6 clusters per muscle spindle (n=15 spindles). (**E** to **H**) Na$_V$1.6 clusters (**G**) colocalize with Ankyrin-G (yellow, **E** and **F** are insets from **G**). (**H**) Quantification of the percentage of Ankyrin G clusters that colocalize with Na$_V$1.6 clusters (n=9 spindles). (**I** to **M**) Co-labeling of Na$_V$1.6 (**J**) with juxtaparanode maker CASPR (**K**, yellow) reveal proprioceptor nodes of Ranvier (**L**) and heminodes (**M**) (n=10 spindles). Nodes of Ranvier were identified by two CASPR+ signals (arrows) flanking Na$_V$1.6 clusters (arrowhead). Heminodes were identified by a single CASPR+ signals juxtaposed to Na$_V$1.6 cluster. (**N** to **S**) Co-labeling of Na$_V$1.6 (**O**) with Na$_V$1.1 (**P**, yellow) show discrete cellular expression patterns. (**Q** to **S**) Arrowhead

454 denotes $Na_V1.6$ channels and arrows denote $Na_V1.1$ channels. N=3-5 mice. Inset scale bars=10 μm.

455 Scale bars=20 μm

456

$Na_V1.1$ and $Na_V1.6$ occupy distinct cellular domains within muscle spindles

458 Our results demonstrate that $Na_V1.1$ and $Na_V1.6$ have differential roles in proprioceptive signaling.

459 We next sought out to define the mechanistic basis of their distinct and nonredundant contributions.

460 At the biophysical level, $Na_V1.1$ and $Na_V1.6$ are functionally very similar, and both contribute to

461 peak, persistent, and resurgent sodium currents (*26–28*). Previous studies examining central

462 neurons, however, show that $Na_V1.1$ and $Na_V1.6$ occupy distinct excitable domains (*29–31*),

463 suggesting that differences in cellular localization could dictate the unique roles these channels play

464 in proprioception. Compared to the central nervous system, our understanding of Na_V expression

465 in sensory terminals is extremely poor. We therefore set out to examine the expression patterns of

466 $Na_V1.1$ and $Na_V1.6$ in proprioceptive end organs. We focused our analysis on muscle spindles, as

467 these structures comprise two of the three proprioceptor functional classes (*1*) and are the afferent

468 endings from which we recorded in *ex vivo* muscle nerve experiments (Figs. 2 and 3). We first

469 labeled for $Na_V1.6$ channels and observed discrete, high density clusters across the spindle that

470 resembled action potential initiation zones (Fig. 9A to C). We observed approximately 2 $Na_V1.6^+$

471 clusters per muscle spindle section (Fig. 9D), though this is likely an underestimation of the total

472 number of clusters per entire spindle. In central neurons, $Na_V1.6$ has been shown to play a major

473 role in signal initiation and propagation due to its expression at the distal axon initial segment (AIS)

474 and nodes of Ranvier (*29, 31, 32*). Thus, we co-labeled with the AIS marker Ankyrin-G (AnkG,

475 Fig. 9 E to G, *33*) and found that 100% of $Na_V1.6$ clusters colocalize with AnkG (Fig. 9 H). To

476 determine if these clusters were *bona fide* heminodes or nodes of Ranvier, we co-labeled with the

477 juxtaparanode marker CASPR (*34*). Indeed, triple immunolabelling experiments found $Na_V1.6$

478 clusters flanked by two CASPR+ signals near VGLUT1$^+$ muscle spindles (Fig. 9 I to L), as well as

479 in myelinated axons of the sciatic nerve (Fig. S8). Furthermore, we also observed $Na_V1.6$ channel

480   clusters flanked by a single CASPR$^+$ signal within muscle spindles, indicative of the presence of

481   Na$_V$1.6$^+$ heminodes (Fig. 9 M). We ensured the specificity of Na$_V$1.1 and Na$_V$1.6 antibodies using

482   tissue harvested from Na$_V$1.1^{cKO} and Na$_V$1.6^{cKO} mice, respectively (Fig. S9). These findings reveal

483   that Na$_V$1.6 is expressed only at heminodes within muscle spindles and nodes of Ranvier of

484   proprioceptors, where it likely plays a direct role in signal initiation and propagation.

485

486   If Na$_V$1.1 and Na$_V$1.6 differentially regulate electrical signaling in proprioceptors through distinct

487   cellular localization patterns, co-labeling for both ion channels should reveal non-overlapping

488   expression patterns. In line with our hypothesis, we find a notable difference in Na$_V$1.1 localization

489   in muscle spindles compared to Na$_V$1.6 (Fig 9 N and Q). In contrast to the discrete clusters of

490   Na$_V$1.6, we observe Na$_V$1.1 localization is broader, but restricted to more equatorial wrappings

491   within muscle spindles and in some presumptive axons entering the muscle spindle (Fig. 9 S).

492

493   Given we found developmentally dependent roles for Na$_V$1.1 and Na$_V$1.6 in proprioceptor synaptic

494   transmission in the spinal cord (Figs. 4 and 5), we asked whether Na$_V$ localization is dynamic during

495   postnatal development. We labeled for Na$_V$1.1 and Na$_V$1.6 in muscle spindles from the EDL of P7

496   and P14 C57Bl6/J mice (Fig. S10), the timepoints at which we observed a change in the requirement

497   for either channel to motor function (Figs. S2 and S4). At P7, we observed no Na$_V$1.6 clusters within

498   spindles, though we did find some clusters near spindles, which could represent nodes of Ranvier.

499   At P14, clusters of Na$_V$1.6 begin to emerge within muscle spindles, though these clusters appear

500   smaller and less frequently than those observed in adult muscle spindles. We detected little to no

501   Na$_V$1.1 immunoreactivity in muscle spindles at P7 and P14, suggesting either no or low expression

502   of this channel at these timepoints. Given the significant change in functional grasping observed in

503   Na$_V$1.1^{cKO} at P14 (Fig. S4E) this would suggest Na$_V$1.1 may serve a role in sensory transmission

504   outside of the muscle spindle at this timepoint in postnatal development. Thus, we propose that the

505 unique localization patterns of $Na_V1.1$ and $Na_V1.6$, which are dynamically regulated during

506 postnatal development, confer the unique contributions of each channel to electrical signaling in

507 proprioceptors.
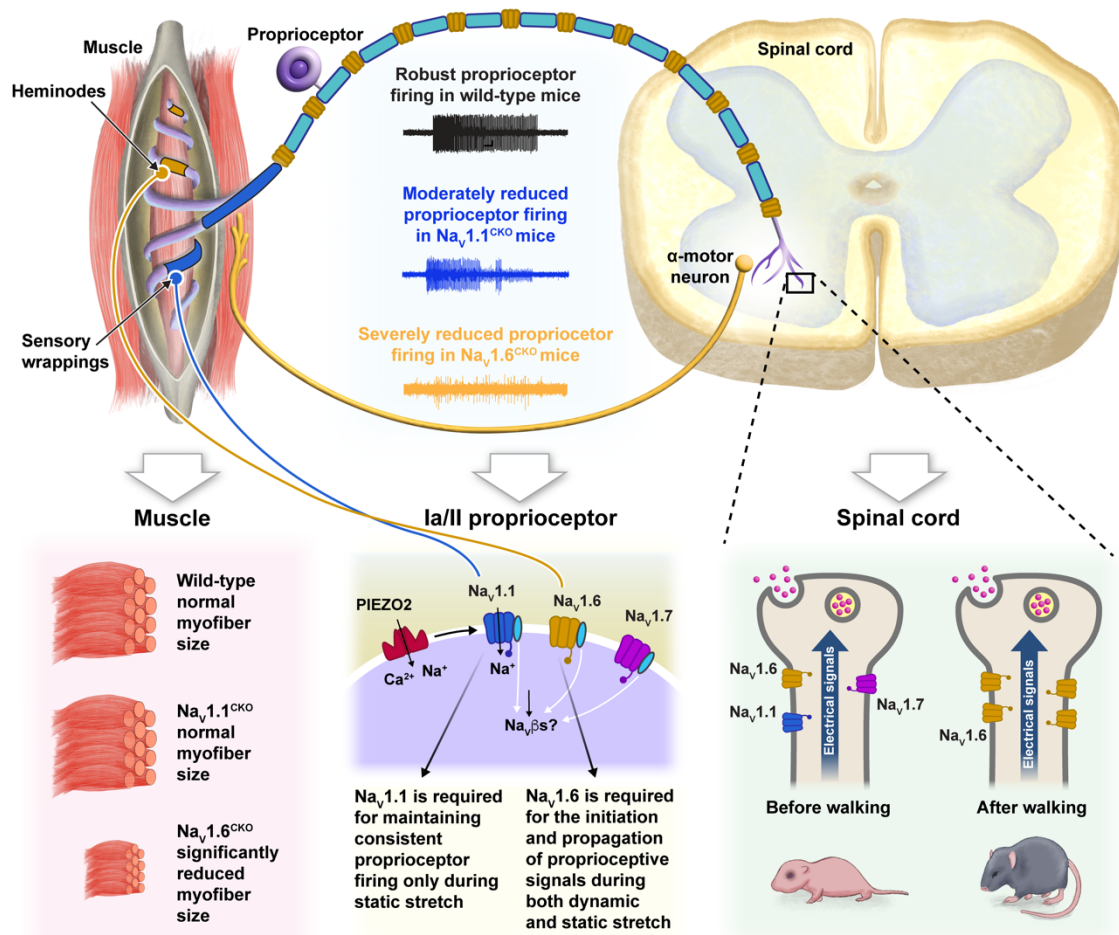
508
509 **Fig. 10. Model of proprioceptive transmission by $Na_V1.1$ and $Na_V1.6$.** Upon muscle stretch,
510 Piezo2 (red) transduces mechanical stimuli into electrical potentials. Following Piezo2 activation,
511 $Na_V1.1$ (blue) expressed in muscle spindle sensory terminals drives consistent proprioceptor firing
512 during static muscle stretch. $Na_V1.6$ (yellow) localized to heminodes and nodes of Ranvier initiate
513 and propagate all proprioceptive signals from muscle spindles to spinal cord. It is likely prior to
514 walking, there is functional redundancy of Na_Vs in proprioceptive axons. After walking behaviors
515 emerge however, proprioceptive synaptic transmission is dependent on $Na_V1.6$. Deletion of $Na_V1.6$
516 in all sensory neurons led to a significant decrease in skeletal muscle fiber size that was not present
517 in $Na_V1.1^{cKO}$ muscle, suggesting that complete loss of proprioceptive feedback non-cell
518 autonomously regulates skeletal muscle development.
519
520

Discussion

The discovery of Piezo2 shared the 2021 Nobel Prize in Physiology and Medicine due to its essential role in the function of various mechanosensory neurons. In proprioceptors, Piezo2 initiates muscle mechanotransduction signaling (*4, 5*); however, the downstream ion channels responsible for transmitting proprioceptive information to central circuits has remained mysterious. Here, we demonstrate that Na$_V$s differentially encode mammalian proprioception, and we predict this is largely due to differences in channel localization within proprioceptors (Fig. 10). Our prior work found that Na$_V$1.1 is essential for maintaining consistent and reliable proprioceptor encoding of static muscle stretch (*7*); this is consistent with its expression at sensory wrappings of muscle spindles where is it poised to amplify Piezo2-mediated mechanotransduction currents. Conversely, due to its localization at heminodes within muscle spindles and nodes of Ranvier, Na$_V$1.6 has an obligate role in initiating proprioceptor action potential firing. This is in line with *ex vivo* recordings from Na$_V$1.6^{cKO} afferents, in which responses to both dynamic and static muscle movement are abolished (Figs. 2 and 3). Furthermore, this demonstrates that the activity of Piezo2 and other ion channels in proprioceptors cannot compensate for the loss of Na$_V$1.6. Thus, we conclude that Na$_V$1.6 is equally essential for mammalian proprioception as Piezo2. To our knowledge, this is the first study to investigate and define unique and nonredundant roles for Na$_V$s in somatosensory encoding.

At the behavioral level, deletion of Na$_V$1.1 or Na$_V$1.6 in sensory neurons led to phenotypically distinct motor deficits. Previously, we reported that Na$_V$1.1^{cKO} mice display uncontrollable intention-like tremors and poor motor coordination (*7*). Here we show that deletion of Na$_V$1.6 in sensory neurons resulted in even more severe ataxia that precluded testing in the rotarod (Fig. 1). Notably, though more impaired than Na$_V$1.1^{cKO} mice, Na$_V$1.6^{cKO} mice did not display intention tremors. The distinct motor phenotypes that result from conditional deletion of Na$_V$1.1 or Na$_V$1.6

547 likely arise from differences in their respective contributions to proprioceptor function and

548 development (Figs. 2, 3 and 6). It should be noted, however, that the behavioral phenotypes in these

549 models cannot solely be attributed to proprioceptor dysfunction, as $Na_V1.1$ and $Na_V1.6$ are also

550 expressed in tactile sensory neurons (*35*), which also contribute to motor behaviors (*36, 37*).

551 Nevertheless, at the afferent level, we show $Na_V1.6$ is fundamentally essential for electrical

552 signaling in proprioceptors, which contrasts the selective impairment on static stretch encoding

553 observed in $Na_V1.1^{cKO}$ mice. Furthermore, $Na_V1.1$ deletion had no significant impact on muscle

554 spindle or skeletal muscle development; therefore, it is likely that the deficits observed in $Na_V1.1^{cKO}$

555 are purely electrical in nature. By contrast, in addition to loss of proprioceptive transmission,

556 $Na_V1.6^{cKO}$ mice had abnormal muscle spindle structure (Fig. 6) and significantly reduced skeletal

557 muscle fiber size (Fig. 7), which suggests that the severe motor deficits in $Na_V1.6^{cKO}$ may be caused

558 by both cell-autonomous and non-cell-autonomous mechanisms. Interestingly, prior work on

559 *Scn8a^{med}* mice show that global inactivation of $Na_V1.6$ resulted in a similar ataxic-like phenotype,

560 as well as muscle atrophy and weakness (*12, 22*). Our data show loss of $Na_V1.6$ function in sensory

561 neurons, particularly proprioceptors, are a major contributor to the motor and muscle impairments

562 in *Scn8a^{med}* mice. This may have broader implications for interpreting the clinical manifestations

563 associated with disease causing *Scn8a* mutations, which often result in motor dysfunction (*11*).

564

565 We found that a single copy of $Na_V1.6$ in sensory neurons was sufficient for normal motor function

566 in adults (Fig. 1), despite being haploinsufficient in *ex vivo* muscle nerve recordings in response to

567 vibration (Figs. 3). Indeed, in response to vibratory stimuli, afferents from $Na_V1.6^{het}$ animals were

568 less likely to entrain to sinusoidal vibration, particularly at $25\mu m$ stimulus amplitudes (Fig. 3 B and

569 D). Why these functional deficits in $Na_V1.6^{het}$ afferents do not manifest at the behavioral level is

570 unclear. It should be noted that at P14 we observed $Na_V1.6$ haploinsufficiency in *ex vivo*

571 monosynaptic reflex recordings (Fig. 4 and Table S1) and in a motor coordination assay (Fig S2).

572 It is therefore possible that compensatory mechanisms in $Na_V1.6^{het}$ animals come into play in early

573 adulthood. Another possibility is that $Na_V1.6^{het}$ animals possess more subtle motor deficits that

574 were unresolvable in the open field and rotarod. More sensitive kinematic analyses with higher

575 spatial and temporal resolution will be required to investigate the extent of $Na_V1.6$

576 haploinsufficiency for proprioceptor-driven motor behaviors.

577

578 We have very limited knowledge about the localization of ion channels within somatosensory end

579 organs. This information is important for understanding how electrical signals arise within

580 structurally complex sensory terminals, which can be damaged during pathological conditions or

581 aging (*1, 38–40*). We show that $Na_V1.1$ and $Na_V1.6$ occupy distinct cellular compartments within

582 proprioceptive muscle spindle end organs, which we predict underlies their differential roles in

583 encoding proprioceptive signals. While differences in biophysical properties could also underly the

584 differential roles of $Na_V1.1$ and $Na_V1.6$ to proprioceptive transmission, these channels share many

585 functional similarities. They both rapidly activate and inactivate, can generate peak, persistent and

586 resurgent currents, and have similar recovery from inactivation kinetics (*7, 27, 28, 41–43*). Prior

587 work in neurons of the central nervous system is consistent with our hypothesis that localization

588 dictates Na_V contributions to proprioceptor function. For example, in retinal ganglion cells and

589 motor neurons, $Na_V1.6$ is preferentially expressed at the distal axon initial segment, suggesting a

590 primary role in signal initiation (*44, 45*). Furthermore, studies have shown that $Na_V1.6$ is the

591 dominant isoform at nodes of Ranvier, playing a key role in action potential propagation in

592 myelinated axons (*31, 45*). In contrast, $Na_V1.1$ is localized to the soma and proximal AIS, where it

593 aids in repetitive firing in fast spiking neurons of the brain (*14, 46*). This is in line with our model

594 whereby $Na_V1.1$ amplifies mechanotransduction currents from Piezo2 to maintain sustained action

595 potential firing during static stretch.

596

597 Interestingly, we found that 100% of $Na_V1.6$ immunoreactivity colocalizes with AnkG. AnkG is

598 known to anchor Na_Vs within the AIS of central neurons (*32, 33, 47, 48*); thus, our findings indicate

599 muscle spindles possess several $Na_V1.6$-expressing action potential initiation zones. Surprisingly,

600 we never observed broad AnkG immunoreactivity in muscle spindle sensory wrappings, indicating

601 AnkG does not colocalize with $Na_V1.1$, despite its known colocalization with $Na_V1.1$ at the AIS in

602 other neurons of the central nervous system (*30, 49*). The mechanisms that anchor $Na_V1.1$ to

603 sensory terminals remain unknown. Scaffolding proteins known to colocalize with $Na_V1.1$ include

604 βIV-spectrin, auxiliary $Na_Vβ$ subunits, and fibroblast growth factors (*14, 50, 51*). Proximity

605 proteomic approaches could identify specific molecular players involved in $Na_V1.1$ channel

606 organization within proprioceptive end organs.

607

608 Another surprising result from our study was the developmentally dependent manner in which Na_Vs

609 contribute to proprioceptor synaptic transmission in the spinal cord (Fig. 10). Ventral root

610 recordings from mice at ages P6 to P11 revealed that neither $Na_V1.1$ or $Na_V1.6$ are required for the

611 proprioceptor-mediated monosynaptic reflex response at this age. Conversely, by P14, when

612 proprioceptors are nearing molecular maturation and weight bearing locomotion has emerged,

613 $Na_V1.6$ becomes absolutely critical for this circuit. There are two principal interpretations for these

614 data. First, prior to walking behaviors, neither $Na_V1.1$ or $Na_V1.6$ contribute to proprioceptor

615 synaptic transmission onto motor neurons. This interpretation would be consistent with previous

616 studies in myelinated neurons of the retina, whereby the onset of eye opening corresponds with a

617 developmental switch from $Na_V1.2$ to $Na_V1.6$ (*44*). It is possible that another Na_V subtype, such as

618 $Na_V1.7$, is the dominant channel in early postnatal development. Alternatively, another

619 interpretation is that in early postnatal development, there is functional redundancy among Na_V

620 subtypes, and loss of one is insufficient to impair synaptic transmission. This is line with a previous

621 study that found the presence of multiple Na_V isoforms in sensory axons as early as P7 (*52*). We

622 favor the latter interpretation because functional redundancy is a common phenomenon in the

623 developing nervous system, and $Na_V1.7$ does not appear to play a significant role in mammalian

624 proprioception. Nevertheless, both interpretations indicate that the cellular trafficking mechanisms

625 governing the stability of each Na_V subtype in this circuit are independent of one another, as loss

626 of $Na_V1.6$ did not result in compensation by other Na_Vs following the onset of weight bearing

627 locomotion. Notably, our analyses of the monosynaptic reflex are consistent with behavioral

628 analyses carried out in P7 and P14 mice, where motor function in P7 $Na_V1.1^{cKO}$ and $Na_V1.6^{cKO}$

629 mice was largely intact but declined by P14 (Figs. S2 and S4). Interestingly, at P7 we observed

630 little-to-no immunoreactivity of $Na_V1.1$ or $Na_V1.6$ in muscle spindles (Fig. S9). By P14, $Na_V1.6$

631 clusters begin to appear within the spindle, while $Na_V1.1$ immunoreactivity remained weak. This

632 suggests temporally distinct regulation of Na_V localization at peripheral end organs compared to

633 central circuits.

634

635 We find that loss of $Na_V1.6$, but not $Na_V1.1$, resulted in disrupted muscle spindle development. This

636 is in line with recent findings that show mechanosensory neuron end organ development is activity

637 dependent (*21*). Interestingly, previous work found deletion of Piezo2 in proprioceptors (Piezo2^{cKO})

638 did not alter muscle spindle structure (*5*); however, these experiments were carried out in 4–5-

639 week-old mice, whereas our analysis of muscle spindle structure was carried out in mice ages 8-12

640 weeks. This raises the possibility that electrical activity is required for the maintenance, but not

641 development, of muscle spindle structure.

642

643 Surprisingly, $Na_V1.6^{cKO}$ mice showed significantly reduced skeletal muscle fiber size, highlighting

644 a potential non-cell-autonomous role for proprioceptive feedback in skeletal muscle development

645 or maintenance (Figs. 7 and 10). This effect was not seen in $Na_V1.1^{cKO}$ mice, but is consistent with

646 a study that found deletion of Piezo2 in proprioceptors led to non-cell-autonomous deficits in spine

647 alignment and hip joint formation (*53*). Despite the smaller size of $Na_V1.6^{cKO}$ skeletal muscle fibers,

648 all $Na_V1.6^{cKO}$ intrinsic muscle properties were similar to $Na_V1.6^{het}$ and $Na_V1.6^{fl/fl}$ mice. Grip

649 strength in $Na_V1.6^{cKO}$ mice, however, was significantly weaker compared to other genotypes. These

650 findings suggest that reduced grip strength in $Na_V1.6^{cKO}$ mice is likely due to impaired motor neuron

651 activation of skeletal muscle and not due to changes in intrinsic muscle function. Interestingly, prior

652 studies have identified dysfunction in proprioceptive spinal cord circuits in mouse models of spinal

653 muscular atrophy and amyotrophic lateral sclerosis (*19*, *54–56*). An intriguing possibility is that

654 loss of proprioceptive feedback onto motor neurons in $Na_V1.6^{cKO}$ mice (Fig. 4) could lead to

655 pathophysiological phenotypes similar to those observed in neuromuscular or motor neuron disease.

656

657 Mutations in the genes that encode $Na_V1.1$ and $Na_V1.6$, *Scn1a* and *Scn8a*, respectively, are strongly

658 associated with neurological diseases in which ataxia and motor developmental delays are

659 prominent clinical manifestations (*12*, *26*, *46*). How proprioceptor dysfunction contributes to these

660 disorders is unknown. The majority of Dravet syndrome patients have complete loss-of-function

661 (LOF) of one copy of *Scn1a*; conversely, patient reported mutational variants in *Scn8a* are

662 predominately gain-of-function (GOF). Our previous work found that proprioceptor afferents from

663 $Na_V1.1^{het}$ mice also had impaired encoding static muscle stretch (*7*), suggesting that motor

664 dysfunction in patients missing one functional copy of *Scn1a* could be in part sensory in nature;

665 however, it is unclear how *Scn8a* GOF mutations would affect proprioceptor function. Patients

666 harboring either *Scn1a* LOF and *Scn8a* GOF mutations have similar motor deficits (*11*); thus, one

667 possibility is that *Scn8a* GOF mutations lead to use-dependent block of action potential firing in

668 proprioceptors, which could contribute to the similar motor phenotypes observed in these different

669 patient populations (*11*). Interestingly, there are a few reported cases of LOF mutations in *Scn8a*

670 that lead to general ataxia (*57*), and our data suggest that proprioceptor dysfunction could contribute

671 to their motor deficits.

672

673 In addition to $Na_V1.1$ and $Na_V1.6$, proprioceptors also express $Na_V1.7$ (*7*). Mice and humans that

674 lack $Na_V1.7$ are insensitive to pain but do not exhibit prominent deficits in motor function, which

675 suggests a limited role of $Na_V1.7$ in mammalian proprioception (*9, 10*). Alternatively, $Na_V1.1$ and

676 $Na_V1.6$ may compensate for the developmental loss of $Na_V1.7$, which occurs in constitutive genetic

677 mouse models and human patients. It is possible that acute deletion of $Na_V1.7$ in proprioceptors

678 could reveal a previously overlooked contribution of the channel to proprioceptor function. Indeed,

679 we found that $Na_V1.7$ channels contribute to roughly one third of the somal whole-cell sodium

680 current in genetically identified proprioceptors (*7*). Thus, a role for $Na_V1.7$ in mammalian

681 proprioception remains enigmatic.

682

683 A current limitation of the present study is the use of a sensory-neuron wide genetic targeting

684 strategy, which makes interpretation of motor behavior and skeletal muscle impairments

685 confounded by the loss of $Na_V1.1$ or $Na_V1.6$ in other sensory neuron populations, namely touch

686 receptors. As mentioned above, deletion of $Na_V1.1$ or $Na_V1.6$ selectively in proprioceptors is not

687 possible with currently available genetic tools, as the access point for Cre-driven deletion,

688 parvalbumin, is also expressed in neurons of brain and spinal cord that are important for motor

689 function. Despite this limitation, the direct role of $Na_V1.1$ and $Na_V1.6$ in proprioceptors was

690 examined at the functional level in *ex vivo* muscle nerve recordings and spinal cord

691 electrophysiology experiments, as well as in the structural analysis of VGLUT1 identified muscle

692 spindles. To investigate the role of Na_V channels with spatial precision, future experiments will

693 require intersectional strategies for selective gene manipulations in proprioceptors.

694

695 Our data demonstrate that $Na_V1.1$ and $Na_V1.6$ play distinct and nonredundant roles in mammalian

696 proprioception. This work is the first to define how Na_Vs uniquely shape somatosensory

697   transmission and is also the first to show that Na$_V$s occupy distinct cellular compartments in sensory

698   neuron end organs. We predict our results are broadly applicable to other sensory neuron

699   populations, namely mechanoreceptors, which also co-express Na$_V$1.1 and Na$_V$1.6. Furthermore,

700   these data have important translational implications for understanding the motor deficits associated

701   with Na$_V$1.1 and Na$_V$1.6 channelopathies.

702

Materials and Methods

Experimental design

Animals

Pirt^{cre} and Scn8a$^{fl/fl}$ mice were a gift from Drs. Xinzhong Dong (Johns Hopkins University, *58*) and Miriam Meisler (University of Michigan, *59*), respectively. Scn1a$^{fl/fl}$ (stock #041829-UCD) were purchased from the UC Davis MMRRC. All mice used are a C57BL/6J background (non-congenic). Genotyping was outsourced to Transnetyx. Animals use was conducted according to guidelines from the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of UC Davis (#23049) and San Jose State University (#990, *ex vivo* muscle recordings.) Na$_V$1.6^{cKO} mice were provided with wet food and hydrogels daily. Mice were maintained on a 12hr light/dark cycle, and food and water were provided ad libitum.

Animal behavior

Motor function was tested using three assays: rotarod, open-field, and grip strength. Behavioral assays were conducted from least to most invasive in the order of open field, grip strength, and rotarod. All behavioral assays were conducted between 8-10 weeks of age and the experimenter was blind to genotype. For the open field, mice were acclimated to the behavior room for 1 h prior testing. The open field apparatus consisted of a white square box with dimensions of 15x15x20 inches. A camera suspended above the open field tracked animal movement for a single 10-minute period using ANY-maze software. Following testing in the open field, mice were transported to a separate behavior room and allowed to acclimate for 1 h before being assayed in the grip strength and rotarod tests. A grip strength apparatus (IITC Life sciences, Woodland Hills, CA) with a metal grate was used. Mice held from the tail were placed on the metal grate and pulled horizontally away from apparatus once all four paws touched the grate. Mice were assayed across 6 trials with 5-

728 minute intervals between trials. A rotarod machine (IITC) that has an accelerating rotating cylinder

729 was used. $Na_V1.6^{cKO}$ mice were excluded from rotarod testing due to severe motor coordination

730 deficits that prevented them from maintaining balance on the cylinder even in the absence of

731 cylinder rotation. The averages of three trials across three consecutive training days were recorded.

732

Ex vivo muscle nerve recordings

734 Detailed methods on *ex vivo* muscle nerve recordings can be found in Wilkinson et al. 2012 (*16*).

735 Briefly, extensor digitorum muscle and innervating peroneal branch of the sciatic nerve were

736 dissected from adult mice and placed in a tissue bath of oxygenated Synthetic Interstitial Fluid at

737 24ºC. Tendons were tied to a fixed post and lever arm of a dual force and length controller and

738 transducer (300C-LR, Aurora Scientific, Inc.). The cut end of the nerve was suctioned into a bipolar

739 glass electrode and connected to an extracellular amplifier with headstage (Model 1800, A-M

740 Systems). Muscles were held at the length of maximal twitch contraction, Lo. For static stretch

741 experiments, nine 4 s ramp-and-hold stretches were given at 2.5, 5, and 7.5% of Lo (Ramp speed

742 was 40% Lo/s). Stretch lengths were repeated three times. For sinusoidal stimuli, sixteen 9 s

743 sinusoidal vibrations were given at 5, 25, 50, and 100 μm amplitudes at varying frequencies (10,

744 25, 50, and 100 Hz). A rest period of 1 min was given between each length change. Resting

745 discharge was quantified as the firing rate 10 s before stretch. Firing rate during the static phase of

746 stretch was calculated 3.25-3.75 sec into the hold phase of stretch (LabChart Software,

747 ADInstruments). The consistency of firing during static muscle stretch was found by calculating

748 the interspike interval coefficient of variation during the plateau phase of stretch (CV = Std

749 Dev/Mean of ISI 1.5 – 3.5s after ramp up). For dynamic responses, the average firing rates during

750 the 9 s vibration was determined. Entrainment was defined as whether a unit could entrain in a 1:1

751 fashion to vibration stimulus. In most afferents we confirmed that they were Group Ia or II afferents

752 by looking for a pause in firing during the shortening phase of contraction (a train of 60 stimulations

753 of 0.5 ms pulse width were given at 1 Hz frequency from a 701C stimulator (Aurora Scientific)).

754

755 **Spinal cord electrophysiology**

756 Spinal cords were harvested from postnatal mice spanning the age-groups P6-11 and P14-18. The

757 mice were deeply anesthetized with isoflurane, decapitated and eviscerated. We followed the

758 protocol that has been used to record motor activity from mice of weight-bearing age using *ex vivo*

759 spinal cord preparations (*60, 61*). In brief, after evisceration, the preparation was pinned to a

760 dissecting chamber and continuously perfused with ice-cold solution, comprising (in mM): 188

761 sucrose, 25 NaCl, 1.9 KCl, 10 $MgSO_4$, 0.5 NaH_2PO_4, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 25 glucose,

762 bubbled with 95 % O_2/ 5 % CO_2. The spinal cord was exposed following a ventral laminectomy

763 and transected at the thoracic levels (T5-T8). The dorsal and ventral roots were isolated over the

764 sixth lumbar segment, bilaterally, just proximal to the dorsal root ganglion. All other dorsal and

765 ventral roots were trimmed, and the entire cord was removed from the vertebral column together

766 with the attached roots and transferred to the recording chamber and continuously superfused with

767 artificial cerebrospinal fluid (aCSF; concentrations in mM): 128 NaCl, 4 KCl, 1.5 $CaCl_2$, 1 $MgSO_4$,

768 0.5 $NaH2PO_4$, 21 $NaHCO_3$, 30 D-glucose) bubbled with 95 % O_2 – 5 % CO_2. A midsagittal

769 hemisection was performed and the spinal hemicords were allowed to equilibrate in aCSF

770 maintained at ambient temperature. After spinal cord isolation, dorsal and ventral roots at the sixth

771 lumbar segment (L6) were placed into suction electrodes. The dorsal root was stimulated with

772 single pulse stimulus, delivered every 30s, over 10 trials. The stimulus was delivered using a

773 stimulus isolator unit (A365, World Precision Instruments) with current pulse amplitudes set at

774 twice the threshold intensity of stimulation (2T, 0.1 ms pulse-width). Extracellular recordings were

775 made at the ventral roots, the signal was filtered between 0.1–5000 Hz, amplified 1000 times

776 (Model 1700, A-M Systems), digitized at 10 kHz using Digidata 1440A, acquired using Clampex

777 software (v11.2, Molecular Devices), and saved on a computer for offline analysis. Stretch reflex

778 parameters were extracted from the signals for each experiment, after averaging over the 10 trials,

779 using Clampfit (v11.2, Molecular Devices).

780

Muscle Mechanics

782 Soleus muscles were prepared for *ex vivo* passive mechanical testing as previously described (*62*).

783 Briefly, 7-0 sutures were cinched at the muscle-tendon of the soleus and EDL muscles. Suture loops

784 were placed on hooks connected to the 300C-LR-Dual-Mode motor arm and force transducer

785 (Aurora Scientific) such that the muscle remained within 28°C oxygenated Ringer's solution.

786 Twitches were induced using a 701C stimulator (Aurora Scientific) across a range of muscle lengths

787 to determine the optimal length for isometric force generation (Lo). The Lo length corresponded to

788 the length between the sutures on either muscle-tendon junction, as measured by calipers.

789 Physiological cross-sectional area (PCSA) was calculated using the muscle length (Lm), mass (m),

790 ratio of fiber length to Lo (Lf/Lo) and standard density of muscle (ρ=1.06 g/cm^3; PCSA =

791 m/Lo*(Lf/Lo)*ρ, (*63*).

792

793 Soleus muscles were subjected to active mechanical testing, which consisted of a series of 24

794 maximum isometric tetani (300 mA, 0.3 ms pulse width, 80 Hz pulse frequency, 800 ms pulse train)

795 with 6 seconds of recovery in between each tetanus. Muscles were then given 300 seconds to

796 recover before a final tetanus with the same parameters. Maximum isometric force was measured

797 during the first, penultimate, and final tetanus protocol. Maximum forces were normalized to PCSA

798 to give isometric specific tension. The highest isometric specific tension measured during each

799 active protocol was reported as the isometric specific tension for each muscle. The percent of force

800 maintained at the penultimate tetanus compared to the initial tetanus was recorded as the percent

801 force post-fatigue. The percent of force maintained at the final tetanus compared to the initial

802   tetanus was recorded as the percent force post-recovery. After active mechanical testing was

803   completed, muscles were removed from the mechanical testing equipment, embedded in OCT, and

804   flash frozen in liquid nitrogen cooled isopentane. Muscles were stored at -70°C until

805   cryosectioning.

806

807   **Tissue Processing.**

808   For muscle spindle immunolabeling experiments, mice were anesthetized using a

809   ketamine/xylazine cocktail and transcardially perfused with PBS followed by 1% PFA. Extensor

810   digitorum longus (EDL) muscle was then dissected in PBS and post-fixed for 30 min then washed

811   in PBS before incubation in 30% sucrose solution overnight at 4C. Following cryoprotection,

812   muscles were embedded in optimal cutting temperature (Fisher #4585) and stored in -80C until

813   sectioning.

814

815   **Immunohistochemistry.**

816   For immunolabeling experiments in muscle spindles, EDL muscles were sectioned (30μm) along

817   the longitudinal axis. Tissue was incubated in blocking solution (0.1% PBS-T/5% normal goat

818   serum in PBS) and the following primary antibodies were used: guinea pig anti-VGLUT1 (1:8000,

819   Zuckerman Institute 1705) and rabbit anti-βIII tubulin (1:3000, Abcam #ab18207). Secondaries are

820   as follows: anti-guinea pig 488 (1:1000, Thermo Fisher, A11073) and anti-rabbit 647 (1:1000,

821   A32733). For muscle fiber typing experiments, soleus cross sections (20μm) were blocked in a

822   solution containing 5% BSA in PBS. The following primary antibodies were diluted and incubated

823   on muscle sections overnight: mouse IgG2b anti-myosin heavy chain type I (1:250, BA-F8, DHSB)

824   and mouse IgG1 anti-myosin heavy chain type IIa (1:250, SC-71, DHSB). Slides were washed in

825   PBS and the following secondaries were diluted in 2% BSA and incubated for 60 minutes: goat

826   anti-mouse IgG2b 488 (1:500, A21141) goat anti-mouse IgG1 555 (1:500 A21127). After

827 secondary antibody incubation slides were washed in PBS and mounted with FluoromountG with

828 DAPI (SouthernBiotech 0100-20). For immunolabeling of Na_Vs the following primary antibodies

829 were used: guinea pig anti-VGLUT1 (1:8000, Zuckerman Institute 1705), rabbit anti-Na_V1.1

830 (2ug/ml, Neuromab A11954), mouse IgG1 anti-Na_V1.6, mouse (6ug/ml, Neuromab K87A/10.2),

831 rabbit anti-Na_V1.6 (1:750, Alomone, ASC009), IgG2a anti-Ankyrin G (6ug/ml, N106/36.1), and

832 chicken anti-neurofilament heavy (1:3000, Abcam ab4680). Secondaries used were as follows: anti-

833 guinea pig 488 (1:1000, Thermo Fisher, A11073), mouse anti-IgG1 555 (1:1000, A21137), mouse

834 anti IgG2a-647 (1:1000, A21240), anti-chicken 594 (1:1000, WA316328). All specimens were

835 imaged in three dimensions on either a Zeiss LSM880 Airsyscan (63x oil objective, 1.4 NA) or

836 Olympus LV3000 (60x oil objective, 1.4 NA) confocal microscope. Images were analyzed using

837 ImageJ software.

838

839 **Analysis of muscle spindle structure.**

840 Disruptions in muscle spindle sensory endings were quantified by colocalizing VGLUT1

841 immunoreactivity with DAPI to calculate a wrapping efficiency index (WEI). Intrafusal muscle

842 fibers are identifiable in skeletal muscle based on mono- and bi-nucleation via DAPI$^+$ staining. To

843 analyze muscle spindle sensory wrappings without bias, we only analyzed sensory wrappings

844 VGLUT1 sensory wrappings that overlapped with DAPI labeling. For example, in ImageJ, regions

845 of interest (ROIs) were drawn around each sensory wrapping. If the ROI did not overlap with DAPI,

846 it was not counted as a sensory wrapping and was excluded from the analysis. The total number of

847 sensory wrappings (n) were counted and normalized to muscle spindle length (l). The following

848 equation was used to calculate the WEI:

849 $$WEI = n/l$$

850

851 **Experimental Design and statistical analysis.**

852 Summary data are presented as mean ± SD, from n cells, afferents, sections, or N animals. All

853 analysis of immunofluorescent images contained at least 3 biological replicates per condition.

854 Investigator was blinded to genotypes during analysis. For all behavioral, electrophysiological, and

855 mechanics experiments the investigator was blind to genotype. To determine differences in

856 entrainment properties between $Na_V1.6^{fl/fl}$, $Na_V1.6^{het}$, and $Na_V1.6^{cKO}$ we used a logistic regression

857 analysis (SPSS), is a statistical model that calculates the log-odds of an event (i.e. entrainment or

858 non-entrainment) as a linear combination of one or more independent variables (vibration

859 frequency, vibration amplitude and genotype). All other statistical testing was carried out using

860 Prism 10.1 (Graphpad software). Statistical differences were determined using parametric tests for

861 normally distributed data and nonparametric tests for data that did not conform to Gaussian

862 distributions or had different variances. Statistical significance in each case is denoted as follows:

863 *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Source files for each figure can be found on

864 Mendeley.

865

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Supplementary Materials for

Differential encoding of mammalian proprioception by voltage-gated sodium channels.

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This PDF file includes:

> Supplementary Methods
> Figs. S1 to S11
> Table S1
> Movies S1 to S2

Other Supplementary Materials for this manuscript include the following:
> Movies S1 to S2

Supplementary Materials

Supplementary Methods

Assessment of motor function during at postnatal day 7 and 14.
Floxed, heterozygous, and conditional knockout P7 and P14 pups from the $Na_V1.1$ and $Na_V1.6$ mouse lines were assayed for motor dysfunction. The experimenter was blind to genotype.

Behavioral assays at postnatal day 7:

Hindlimb foot angle: In a clear empty mouse cage, a camera was positioned from below and above to record the pup as it moved around the cage. The pup was gently prodded by touching its tail to motivate the pup to move. An average of three pictures were taken from above and below. Using the acquired pictures, measures of the foot angle of the pups were performed using Fiji ImageJ software by drawing a line from the end of the heel/shin to the tip of the middle toe on each hindlimb and measuring the angle of the intersecting lines. Measurements were only taken when the pup was performing a full stride in a straight line and both feet were flat on the ground. Three to five sets of foot angles were measured per pup and used to calculate the average angle for each pup tested.

Righting reflex: Pups were placed on their backs on a bench pad and held in that position for 5 seconds. The pups were released, and the time it took for the pup to return to the prone position was recorded. This was repeated for a total of three trials and the average righting reflex latency was calculated. Intertrial rest periods were 60 seconds.

Hindlimb strength: Using a 50ml conical tube with cotton ball padding at the bottom, the pup was gently placed face down into the tube with its hind limbs hung over the rim. The latency for the pup to fall into the tube was recorded. The test was ended at 60 seconds if the pup did not fall. Each pup was only tested one time to avoid exhaustion.

Grasping reflex: Pups were held by the scruff of the neck in a similar way to the how it is carried by the mother. The pad of each individual mouse paw was stroked using the wooden stick of a cotton tip applicator. The grasp reflex was determined present if the mouse paw curled around the wooden stick. Mice received a score of zero if all four paws had the grasp reflex present, a score of 1 if one paw did not have the grasp reflex present, a score of 2 if two paws did not have the grasp reflex present, and score of 3 if three paws did not have the grasp reflex present, and a score of 4 if four paws did not have the grasp reflex present.

Behavioral assay at postnatal day 14
Modified limb coordination assay: This test was used to determine differences in grip, balance and limb coordination at postnatal day 14. Pups were placed on a wire grid with metal poles running parallel to each other, approximately 8 millimeters apart with a diameter of 3 millimeters. The pups were left on the grid for five seconds and were scored by their ability to grip and balance with each individual limb without the paw slipping in between the metal bars. Pups that could grip/balance with all four limbs received a score of 0, pups that could grip/balance with only three limbs received a score of 1, pups that could grip/balance with only two limbs received a score of 2, Pups that could grip/balance with only one limb received a score of 3, and pups that could grip/balance with none of their limbs received a score of 4. The assay was repeated a total of three times with 30 seconds in between tests, and the average of the three trials was reported.

Multiplex *in situ* hybridization. DRG were harvested from adult (10-15 week-old) Pirt^Cre;Na_V1.6^fl/fl mice of both sexes. DRG was sectioned at 25 μm sections and were processed for RNA *in situ* detection using a modified version of manufacture protocol (Advanced Cell Diagnostics) as previously described (Griffith 2019 and Espino 2022). The following probes were used: Pvalb (421931C1, mouse) and Runx3 (451271-C3, mouse). Following in situ hybridization, sections were incubated in blocking solution (5% NGS, 0.1% PBS-T) for 1hr at room temperature (RT). Tissue was incubated in rabbit βIII-Tubulin primary antibodies (1:3000, Abcam ab41489) overnight at 4°C overnight. Tissue was treated with anti-rabbit 594 (1:1000, Invitrogen, A11037) secondary antibodies for 30 min at RT. Sections were mounted with Fluoromount-G with DAPI and imaged in three dimensions on Olympus confocal (FV3000) using 40x 0.90 NA water objective lens. Images were analyzed using ImageJ software.

Immunolabeling of muscle spindles in postnatal development. Immunohistochemistry of EDL harvested from P7 and P14 C57Bl6/J mice was performed. EDL was sectioned (25 μm) on a cryostat and sections were labeled using the following primary antibodies: rabbit polyclonal $Na_V1.1$ (2μL/mL, Neuromab, NACH AP11954), guinea pig anti-VGLUT1 (1:8000, Zuckerman Institute, 1705), and chicken anti-NFH (1:3000, Abcam, ab4680), rabbit polyclonal anti-$Na_V1.6$ (1:750, Alomone Labs, ASC-009). Secondary antibodies used were as follows: anti-rabbit 594 (1:500, Thermo Fisher, A32740), anti-guinea pig 647 (1:1000, Thermo Fisher, A11073), and anti-chicken 647 (Thermo Fisher,

1165   A32733), anti-mouse IgG2a 555 (A21137). Specimens were mounted with Fluoromount-G with DAPI
1166   (SouthernBiotech, 0100-20). All specimens were imaged in three dimensions on an Olympus FV3000 confocal
1167   microscope using 60x NA 1.4 oil objective lens. Images were analyzed using ImageJ software.

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1169   **Supplementary figure and legends**
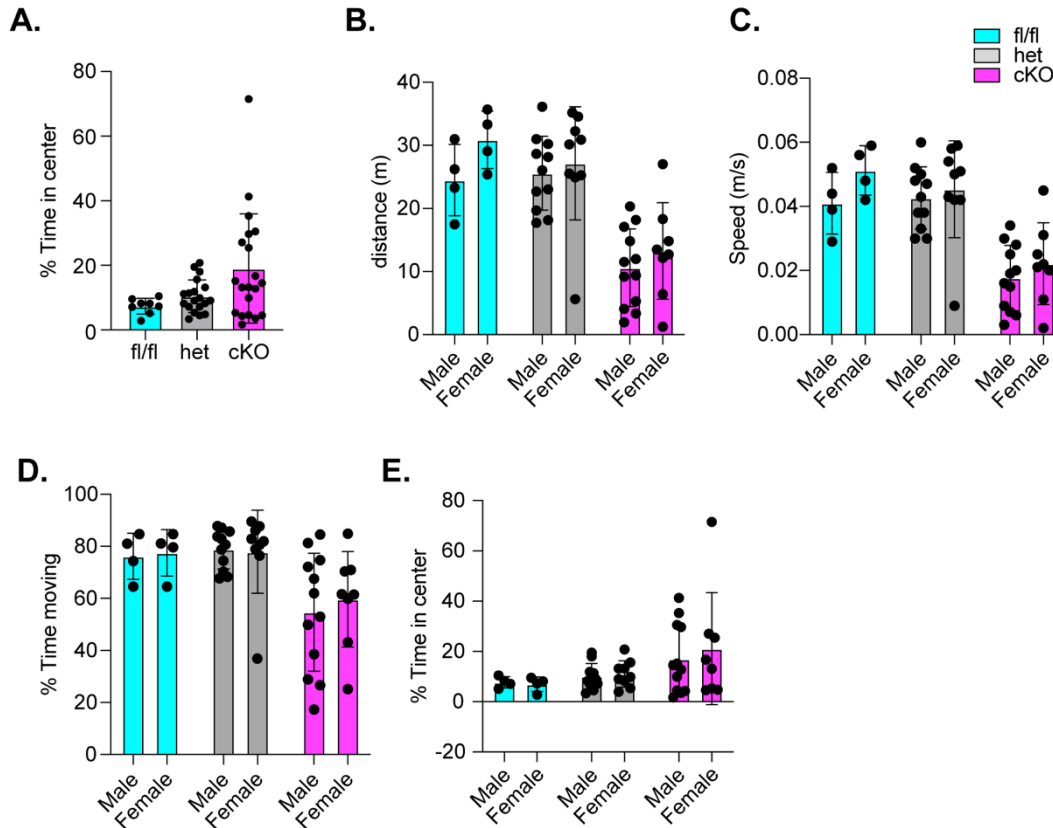
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1171   **Fig. S1. Motor behavior analysis of mice lacking $Na_V1.6$ in sensory neurons**
1172   (**A**) Quantification of percent time spent in the center during a 10 min open-field trial. A Kruskal-
1173   Wallis test with Dunn's Post-hoc comparison was used to determine statistical significance.
1174 $Na_V1.6^{het}$ p=0.4206, $Na_V1.6^{cKO}$ p=0.1026 compared to $Na_V1.6^{fl/fl}$. $Na_V1.6^{fl/fl}$ n = 8, $Na_V1.6^{het}$ n=20,
1175 $Na_V1.6^{cKO}$ n =20. No significant differences were observed for motor behaviors male and female
1176   mice of all genotypes. (**B**) Distance moved. (**C**) Speed. (**D**) Percent time moving. (**E**) Percent time
1177   spend in center.
1178

1179 **Fig. S2. Na$_V$1.6 is required in sensory neurons for motor behaviors in a developmentally dependent manner.**
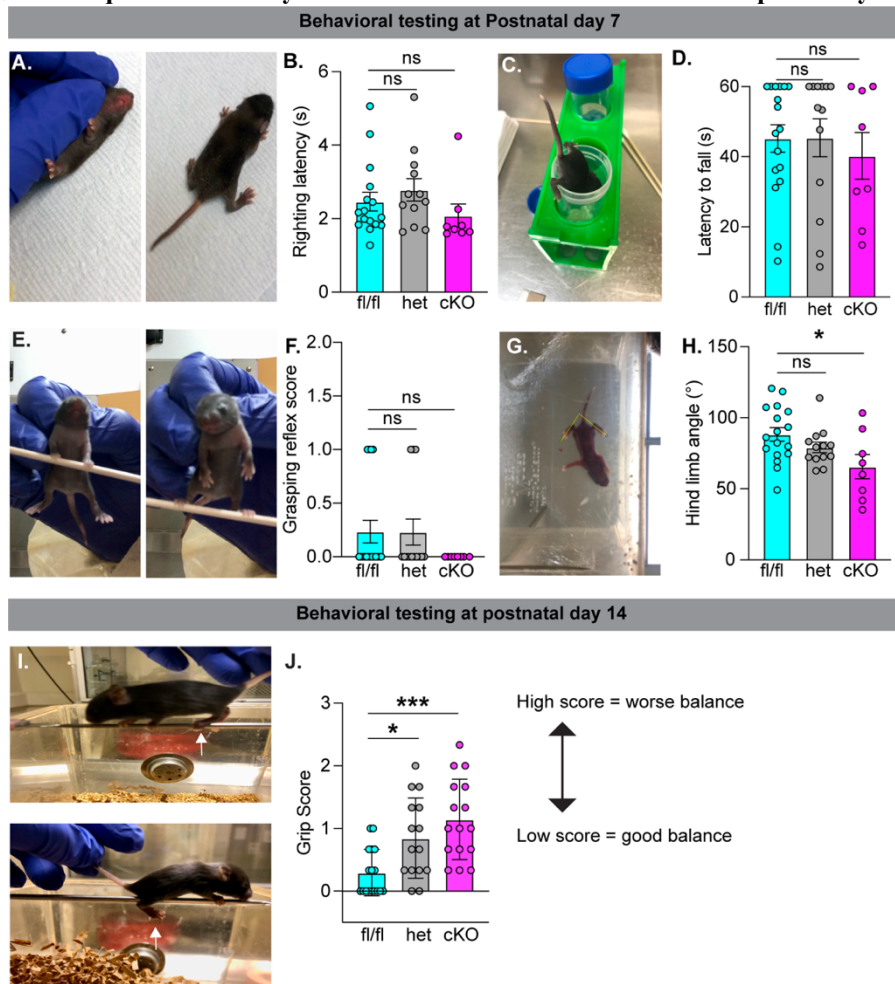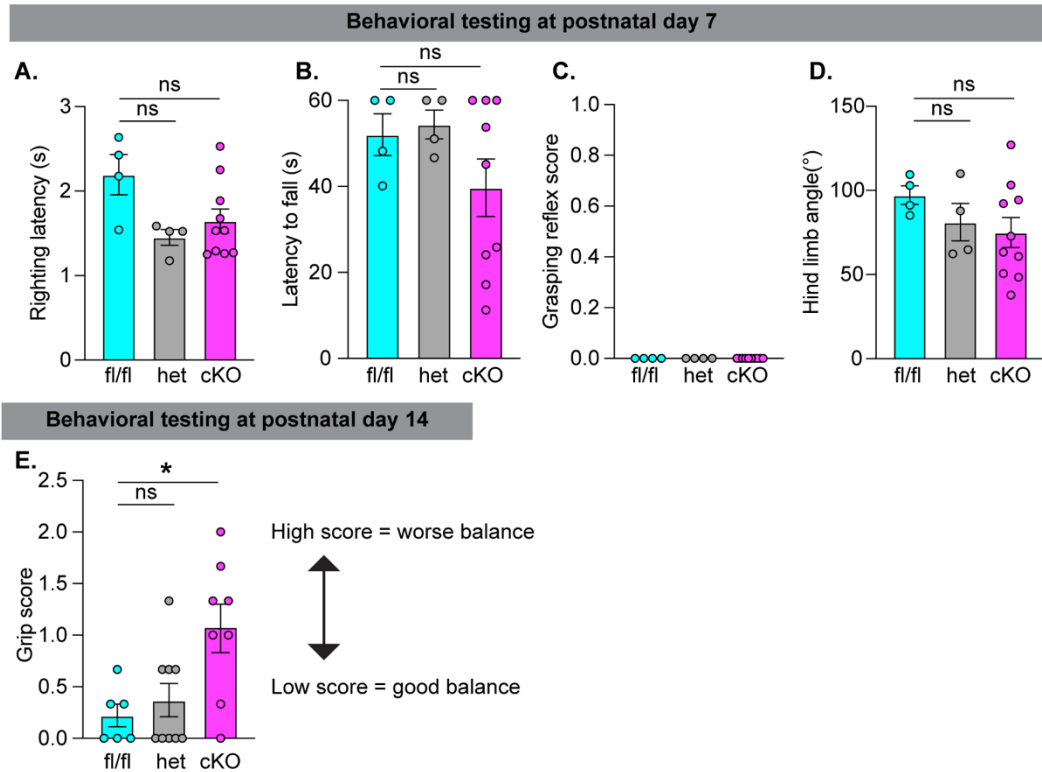

1180 (**A** to **H**) Behavioral testing on mice age P7, N=17 Na$_V$1.6$^{fl/fl}$, N= 13 Na$_V$1.6^{het}, and N= 8 Na$_V$1.6^{cKO}.
1181 (**A**) Representative images of righting reflex before (left) and after (right). (**B**) Latency for mice to
1182 right themselves was quantified. Na$_V$1.6^{het} (grey, p=0.686), Na$_V$1.6^{cKO} (magenta, p=0.655)
1183 compared to Na$_V$1.6$^{fl/fl}$ (cyan). (**C**) Representative image of hind limb strength assay. (**D**) Latency
1184 to fall was quantified. Na$_V$1.6^{het} (p=0.999), Na$_V$1.6^{cKO} (p=0.797) compared to Na$_V$1.6$^{fl/fl}$. (**E**)
1185 Representative images of grip reflex assay on forelimbs (left) and hindlimbs (right). (**F**) Mice were
1186 assayed on grip reflex. Na$_V$1.6^{het} (p=0.999), Na$_V$1.6^{cKO} (p=0.351) compared to Na$_V$1.6$^{fl/fl}$. (**G**)
1187 Representative image of hind limb angle quantification. (**H**) Quantification of mean hindlimb angle.
1188 Na$_V$1.6^{het} (p=0.389), Na$_V$1.6^{cKO} (p=0.021) compared to Na$_V$1.6$^{fl/fl}$. Behavioral testing on mice age
1189 P14. N=17 Na$_V$1.6$^{fl/fl}$, N=15 Na$_V$1.6^{het}, and N=16 Na$_V$1.6^{cKO} (**I**) Representative images from limb
1190 coordination assay. Mice were scored based their ability to grasp the metal grate (top picture shows
1191 successful grasp, bottom picture shows foot slip). (**J**) Quantification of limb coordination score in
1192 P14 mice. N=17 Na$_V$1.6$^{fl/fl}$, N= 17 Na$_V$1.6^{het}, and N= 16 Na$_V$1.6^{cKO}. Na$_V$1.6^{het} (p=0.022), Na$_V$1.6^{cKO}
1193 (p=0.0002) compared to Na$_V$1.6$^{fl/fl}$. A one-way ANOVA with Tukey's Post-hoc comparison was
1194 used to determine statistical significance.
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1197
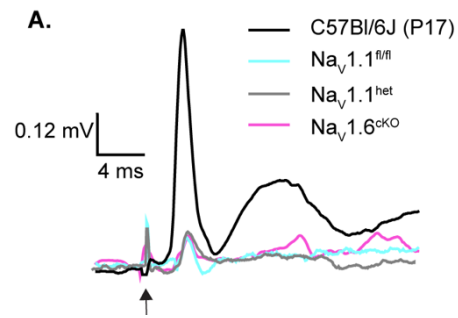
1198 **Fig. S3. Recordings from Pirt^{Cre};Na$_V$1.1 mice at late postnatal development** (**A**) P14 to P18
1199 monosynaptic responses from Na$_V$1.1$^{fl/fl}$ (cyan), Na$_V$1.1^{het} (grey), Na$_V$1.1^{cKO} (magenta), and
1200 C57Bl/6J (black). Recordings from conditional mouse line exhibited small monosynaptic responses
1201 compared to age matched C57Bl/6J mice that were not reliably quantifiable.

Fig. S4. $Na_V1.1$ in sensory neurons is required for motor function in late postnatal development. (A to **D)** Behavioral testing on mice age P7. N=4 $Na_V1.1^{fl/fl}$, N= 4 $Na_V1.1^{het}$, and N= 9-10 $Na_V1.1^{cKO}$. (**A**) Latency for mice to right themselves was quantified. $Na_V1.1^{het}$ (grey, p=0.058), $Na_V1.1^{cKO}$ (magenta, p=0.142) compared to $Na_V1.1^{fl/fl}$ (cyan). (**B**) Latency to fall was quantified. $Na_V1.1^{het}$ (p>0.999), $Na_V1.1^{cKO}$ (p=0.715) compared to $Na_V1.1^{fl/fl}$. (**C**) Mice were assayed on grip reflex. All measured values between genotypes. (**D**) Quantification of mean hindlimb angle. $Na_V1.1^{het}$ (p=0.637), $Na_V1.1^{cKO}$ (p=0.906) compared to $Na_V1.1^{fl/fl}$. (**E**) Quantification of limb coordination score in P14 mice. N=6 $Na_V1.1^{fl/fl}$, N= 9 $Na_V1.1^{het}$, and N= 8 $Na_V1.1^{cKO}$. $Na_V1.1^{het}$ (p=0.845), $Na_V1.1^{cKO}$ (p=0.015) compared to $Na_V1.1^{fl/fl}$. A one-way ANOVA with Tukey's Post-hoc comparison was used to determine statistical significance.

1213 **Fig. S5. The number of proprioceptors in DRG is unaffected due to loss of Na$_V$1.6 in sensory neurons.**
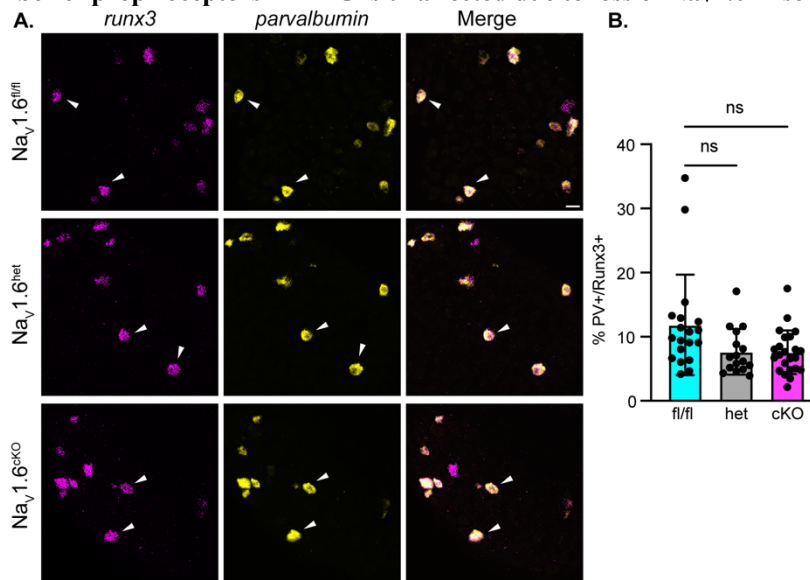
1214 (**A**)Representative confocal images of Na$_V$1.6$^{fl/fl}$ (**top**), Na$_V$1.6^{het} (**middle**), and Na$_V$1.6^{cKO} (**bottom**)
1215 adult dorsal root ganglion (DRG) neuron section (25μm). Sections were hybridized with probes
1216 targeted against parvalbumin (Pvalb, yellow) and Runx3 (magenta). (**B**) Quantification of the
1217 percentage of Pvalb+/Runx3+ neurons per genotype. Each dot represents a single DRG section.
1218 Images were acquired with a 40x, 0.9 NA water immersion objective. A Kruskal-Wallis test with
1219 Dunn's Post-hoc comparison was used to determine statistical significance. Na$_V$1.6^{het} p=0.0694,
1220 Na$_V$1.6^{cKO} p=0.0511 compared to Na$_V$1.6$^{fl/fl}$. N=3 mice for each genotype. Na$_V$1.6$^{fl/fl}$ n = 19,
1221 Na$_V$1.6^{het} n=16, Na$_V$1.6^{cKO} n =22 sections.
1222
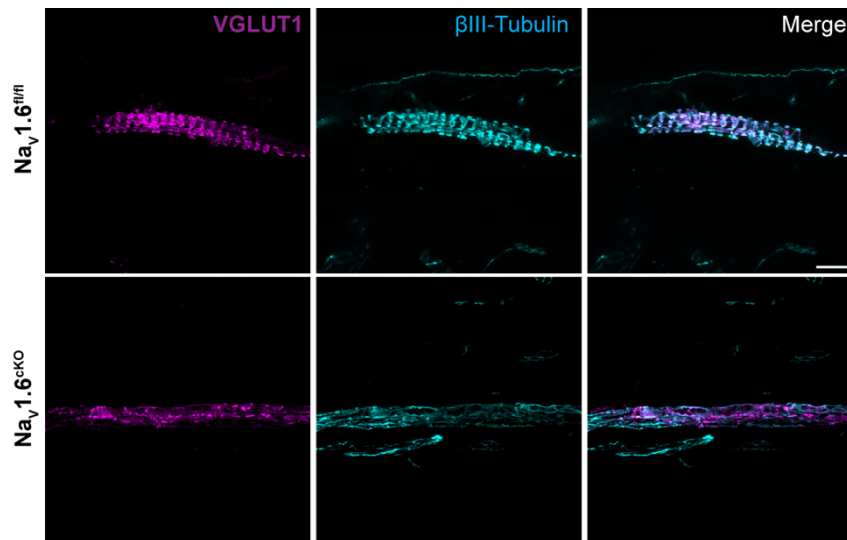
Fig. S6. βIII-Tubulin and VGLUT1 labeling in muscle spindles are similar in mice Na$_V$1.6$^{fl/fl}$ and Na$_V$1.6^{cKO} mice.
Representative confocal images of muscle spindles from Na$_V$1.6$^{fl/fl}$ (**top**) and Na$_V$1.6^{cKO}(**bottom**) adult extensor digitorum longus muscle. Muscle spindle afferents are labeled were colabeled with VGLUT1 (magenta) and βIII-Tubulin (cyan) antibodies. Images were acquired with 60x, 1.4 NA oil immersion objective. Scale bar set to 25 μm.
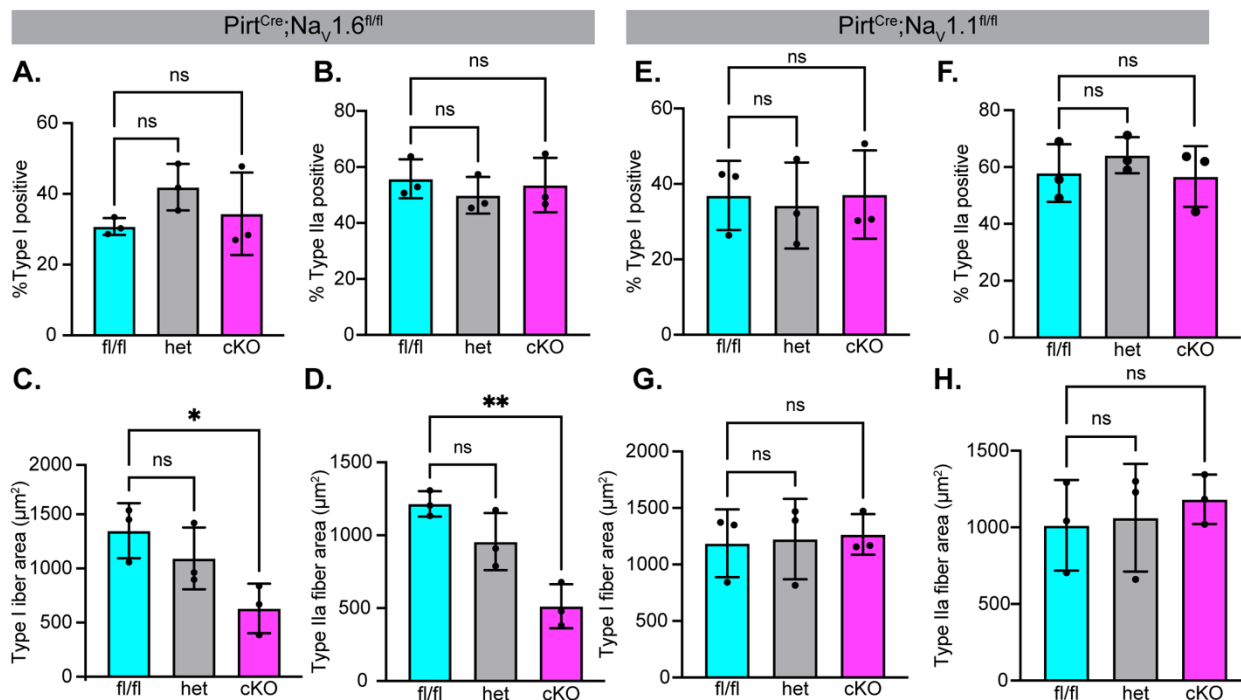
Fig. S7. Muscle fiber type is unaffected due to loss of $Na_V1.6$ or $Na_V1.1$ in sensory neurons
(**A** to **D**) Quantification of average muscle fiber type in $Na_V1.6^{fl/fl}$ (cyan), $Na_V1.6^{het}$ (grey), and $Na_V1.6^{cKO}$ (magenta). (**A**) Percentage of type I positive muscle fibers, $Na_V1.6^{het}$ p=0.2217, $Na_V1.6^{cKO}$ p=0.8060 compared to $Na_V1.6^{fl/fl}$. (**B**) Type II positive muscle fibers, $Na_V1.6^{het}$ p=0.5861, $Na_V1.6^{cKO}$ p=0.9170 compared to $Na_V1.6^{fl/fl}$. (**C**) Type I fiber area, $Na_V1.6^{het}$ p=0.4209, $Na_V1.6^{cKO}$ p=0.0250 compared to $Na_V1.6^{fl/fl}$. (**D**) Type II fiber area, $Na_V1.6^{het}$ p=0.1376, $Na_V1.6^{cKO}$ p=0.0023 compared to $Na_V1.6^{fl/fl}$. (**E** to **H**) Quantification of average muscle fiber type in $Na_V1.1^{fl/fl}$ (cyan), $Na_V1.1^{het}$ (grey), and $Na_V1.1^{cKO}$ (magenta). (**E**) Percentage of type I positive muscle fibers, $Na_V1.1^{het}$ p=0.9358, $Na_V1.1^{cKO}$ p=0.9995 compared to $Na_V1.1^{fl/fl}$. (**F**) Type II positive muscle fibers, $Na_V1.1^{het}$ p=0.9662, $Na_V1.1^{cKO}$ p=0.6959 compared to $Na_V1.1^{fl/fl}$. (**G**) Type I fiber area, $Na_V1.1^{het}$ p=0.9819, $Na_V1.1^{cKO}$ p=0.9236 compared to $Na_V1.1^{fl/fl}$. (**H**) Type II fiber area, $Na_V1.1^{het}$ p=0.6435, $Na_V1.1^{cKO}$ p=0.9799 compared to $Na_V1.1^{fl/fl}$. A one-way ANOVA with Dunn's post hoc comparison was used to determine statistical significance. Each dot represents a single animal. N=3 for each genotype.
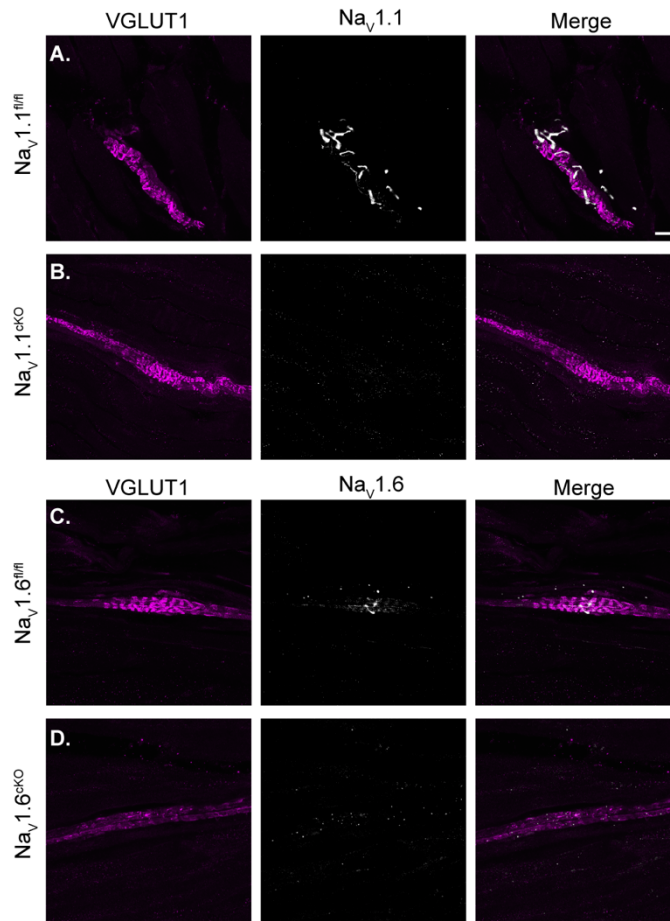
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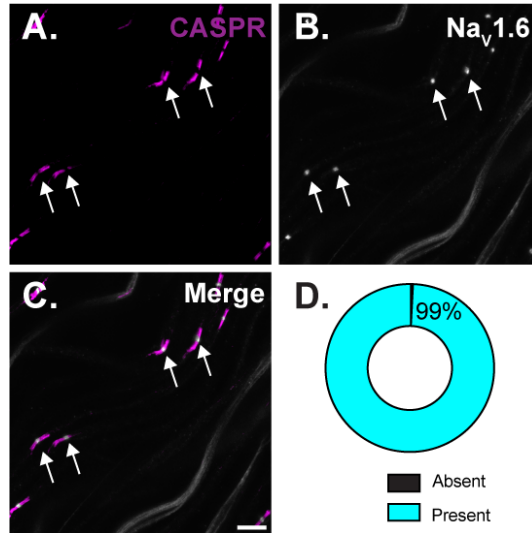
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1248 **Fig. S8. Na$_V$1.6 highly expressed at nodes of Ranvier sensory axons.** (**A** to **C**) Representative images for sensory
1249 nodes of Ranvier were identified via CASPR (**A**) and Na$_V$1.6 (**B**) immunoreactivity. (**D**) Quantification of the
1250 percentage of nodes of Ranvier that express Na$_V$1.6. n=247 nodes, N=3 mice. Scalebar=10μm

Fig. S9. Validation of Na$_V$ antibodies targeting Na$_V$1.1 and Na$_V$1.6

Representative confocal images of muscle spindles from Na$_V$1.1$^{fl/fl}$ (**A**), Na$_V$1.1^{cKO}(**B**), Na$_V$1.6$^{fl/fl}$ (**C**) and Na$_V$1.6^{cKO} (**D**) adult extensor digitorum longus muscle. VGLUT1 (magenta) labels muscle spindle sensory endings. Grey scale represents corresponding Na$_V$ channel isoform. Scale bar set to 20 μm. Images were acquired with a 60x, 1.4 NA oil immersion objective.

1257 **Fig. S10. Na$_V$ labeling in muscle spindles throughout postnatal development**
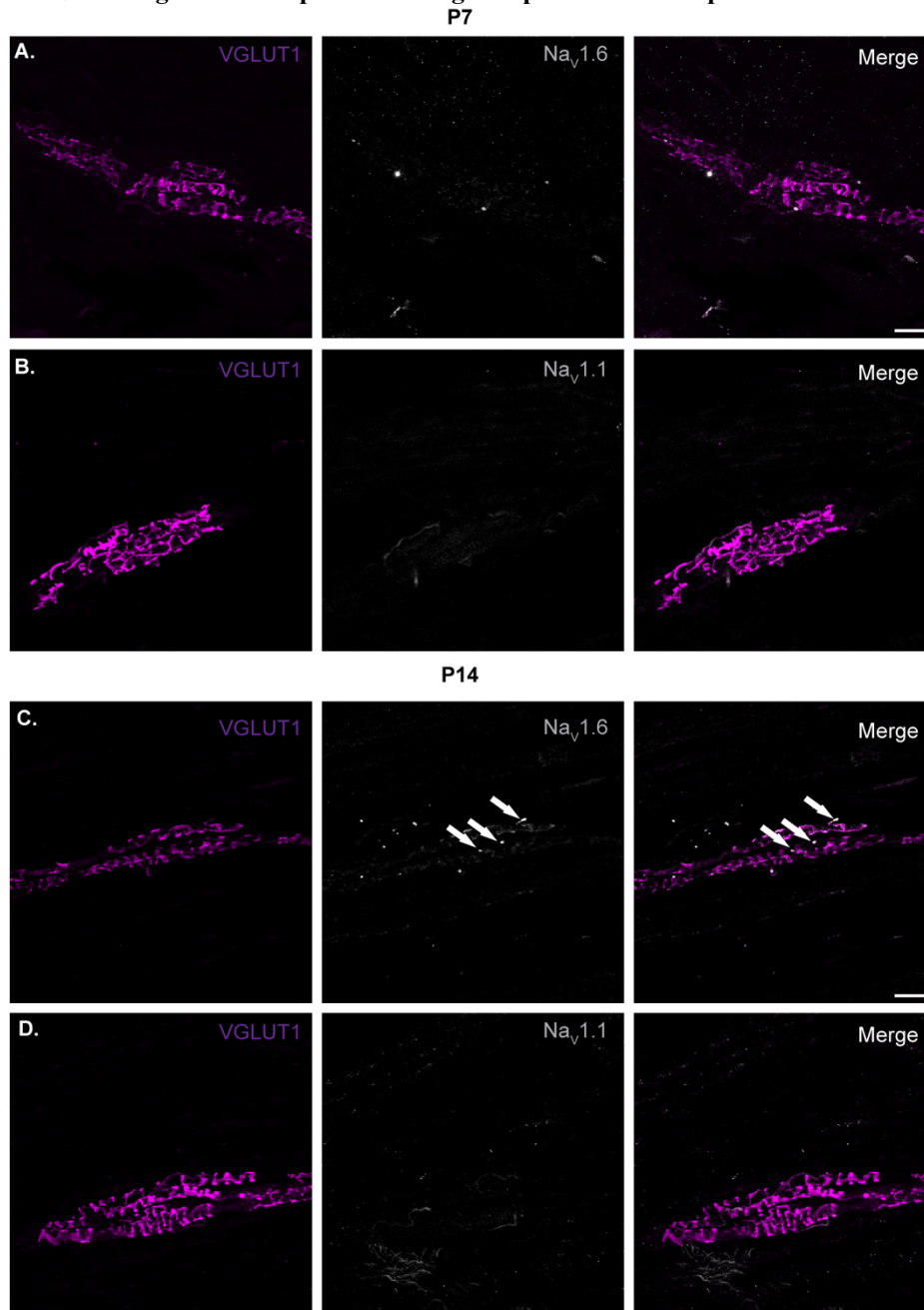
1258 Representative confocal images of Na$_V$1.6 (**A**, 3= mice, 4= spindles) and Na$_V$1.1 (**B**, 4= mice, 6= spindles) labeling in
1259 muscle spindles from extensor digitorum longus muscle from mice at postnatal day 7. Images of Na$_V$1.6 (**C**, 3= mice,
1260 7= spindles, arrows denote clusters of Na$_V$1.6) and Na$_V$1.1 (**D**, 7= mice, 16= spindles) in mice at postnatal day 14.
1261 Images were acquired with a 60x, 1.4 NA oil immersion objective. All tissue was collected from C57BL/6J mice.
1262 Scalebar=25 μm.
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1264   **Supplementary Table**

|	P6-P11 vs. P14-P18		
	$Na_V1.6^{fl/fl}$	$Na_V1.6^{het}$	$Na_V1.6^{cKO}$
Latency	0.019**	0.0515	0.8039
Threshold	0.3536	0.2799	0.0027**
Monosynaptic peak amplitude	0.6501	<0.0001****	0.0027**
Polysynaptic peak amplitude	0.1764	0.0002***	0.0032**
FWHM	0.1848	0.8767	0.0002***

1265   **Table S1: Within-genotype analysis of the monosynaptic reflex response in the $Na_V1.6$**
1266   **mouse line across postnatal development**. P-values obtained from within genotype, across
1267   development, statistical analyses (Two-way ANOVA) of the monosynaptic reflex response in
1268 $Na_V1.6^{fl/fl}$, $Na_V1.6^{het}$, and $Na_V1.6^{cKO}$ mice.
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1273 **Supplementary Movies**
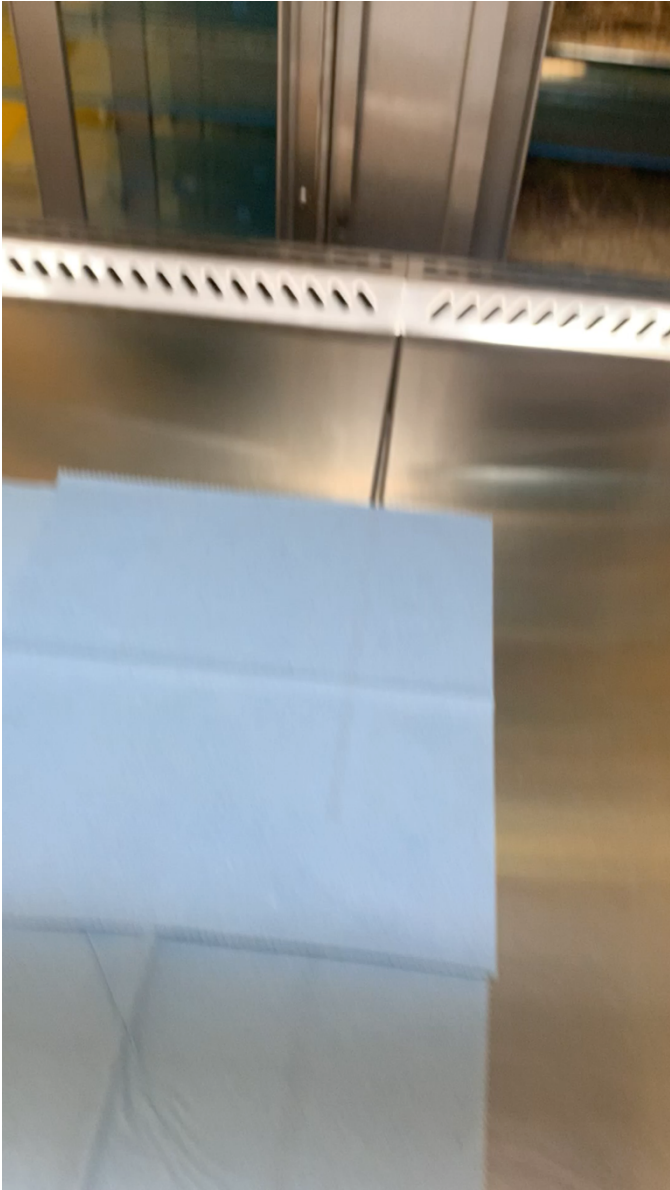
1274 **Movie S1.** Complete loss of limb coordination in $Na_V1.6^{cKO}$ mice when suspended by tails.
1275
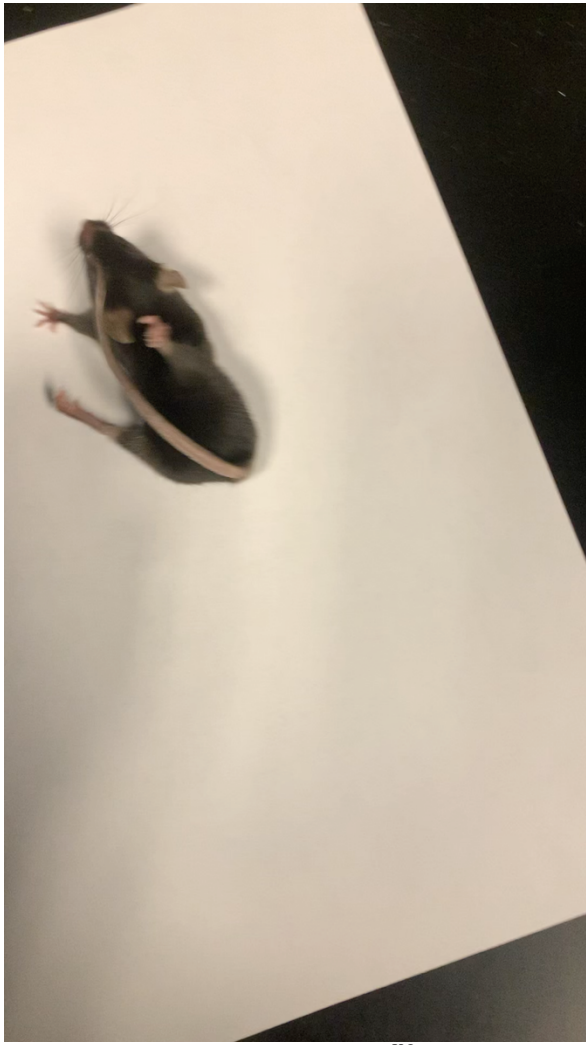
1276   **Movie S2.** An example of a Nav1.6^{cKO} mouse unable hindlimbs or tail to for normal walking behaviors.

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