Skeletal muscle sodium channel gating in mice deficient in myotonic dystrophy protein kinase

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Myotonic dystrophy, a progressive autosomal dominant disorder, is associated with an expansion of a CTG repeat tract located in the 3′-untranslated region of a serine/threonine protein kinase, DMPK. DMPK modulates skeletal muscle Na channels in vitro, and thus we hypothesized that mice deficient in DMPK would have altered muscle Na channel gating. We measured macroscopic and single channel Na currents from cell-attached patches of skeletal myocytes from mice heterozygous (DMPK+/–) and homozygous (DMPK–/–) for DMPK loss. In DMPK+/– myocytes, Na current amplitude was reduced because of reduced channel number. Single channel recordings revealed Na channel reopenings, similar to the gating abnormality of human myotonic muscular dystrophy (DM), which resulted in a plateau of Na current. The gating abnormality deteriorated with increasing age. In DMPK–/– muscle there was reduced Na current amplitude and increased Na channel reopenings identical to those in DMPK+/– muscle. Thus, these mouse models of complete and partial DMPK deficiency reproduce the Na channel abnormality of the human disease, providing direct evidence that DMPK deficiency underlies the Na channel abnormality in DM.

INTRODUCTION

Myotonic muscular dystrophy (dystrophia myotonica, DM), the most common muscular dystrophy in adults, is an autosomal dominant multisystem disease with a prominent abnormality of membrane excitability. The major symptoms are myotonia (the inability to relax a contracted muscle group), progressive skeletal muscle weakness and cardiac conduction disturbances (1,2). The genetic abnormality is the amplification of a CTG repeat tract located in the 3′-untranslated region of the DMPK gene encoding a novel kinase, DMPK, in a gene-rich region of chromosome 19q13.3 (3–7).

The length of the repeat correlates with the severity of the disease (8,9), but the mechanism whereby repeat expansion results in DM is not completely understood. Currently, three non-exclusive models have been proposed. First, transcriptional silencing of one or more genes in the vicinity of the repeat tract could occur due to changes in chromosome topology (10–13). Second, a trans-effect of the repeat expansion at the level of either DNA or RNA might contribute by binding and titration of specific CTG/CUG repeat binding proteins (14–16). Third, repeat expansion may result in decreased DMPK levels, occurring as a consequence of abnormal transcription and/or transport of the mutant DMPK message into the cytoplasm (13,17–19).

To test the role of haploinsufficiency of DMPK in the etiology of DM, we and others have developed DMPK-deficient mice in which dmpk has been functionally inactivated (20,21). The resulting mice show a partial DM phenotype characterized by skeletal muscle weakness that occurs as a consequence of abnormal excitation–contraction coupling (21,22). DMPK loss also results in cardiac conduction disorders which are reminiscent of DM patients (23,24). Consistent with human DM, both the cardiac and skeletal muscle pathology were observed to increase progressively as the mutant DMPK animals aged (21).

Several lines of evidence demonstrate that altered modulation of Na channels may play an important role in the pathogenesis of DM. First and most directly, Franke et al. (25) found repeated action potentials and abnormal gating of Na channels in skeletal muscle biopsies of patients with DM. Other indirect lines of evidence link DMPK with Na channel gating. First, co-expression of DMPK alters Na currents in Xenopus oocytes injected with Na channel mRNA (26,27). Second and most importantly, Benders et al. (22) noted that the abnormally elevated resting skeletal muscle intracellular calcium concentration in DMPK-deficient mice is corrected by tetrodotoxin, a specific Na channel blocker. This result suggested the possibility that DMPK loss may cause skeletal muscle weakness through an alteration in calcium homeostasis due to changes in Na currents.

To test the idea that DMPK regulates muscle sodium channels in vivo, we have measured membrane potentials and sodium currents in skeletal muscle isolated from mice with complete and partial deficiency of DMPK. As the effects of DMPK deficiency at the whole-organ level are more apparent with increasing age (21,28), we have made measurements in muscle isolated from both young and old mice. We find that DMPK-deficient mice have altered Na channel gating, with reopenings leading to persistent depolarizing current. This effect may contribute to...
the alterations in excitation–contraction coupling, and thus to muscle weakness.

RESULTS

Effects of partial and complete DMPK deficiency on skeletal muscle membrane potentials

Intracellular membrane recordings (Fig. 1A) showed repetitive action potentials in DMPK –/– muscle induced by a single stimulus. These were blocked by lidocaine, a Na channel blocking agent, and were not observed in wild-type mice. Table 1 shows pooled data. Compared with wild-type muscle, bursts of two or more action potentials occurred significantly more commonly in both DMPK –/– and DMPK+/– muscle, but there was no significant difference between DMPK–/– and DMPK+/– muscle. In comparison with wild-type muscle, both DMPK –/– and DMPK+/– muscle were depolarized (Table 1), an effect that was more apparent with increasing age.

Table 1. Repetitive action potentials and resting membrane potential in isolated mouse skeletal myocytes

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Cells with multiple action potentials</th>
<th>Animals</th>
<th>Mean $E_m$ (SD) (mV)</th>
<th>Animals</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0/33</td>
<td>3</td>
<td>–79 (7)</td>
<td>3</td>
<td>37</td>
</tr>
<tr>
<td>Young DMPK+/–</td>
<td>–</td>
<td>–</td>
<td>–74 (4)</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Intermediate-age DMPK+/–</td>
<td>–</td>
<td>–</td>
<td>–70 (8)*</td>
<td>4</td>
<td>71</td>
</tr>
<tr>
<td>Old DMPK–/–</td>
<td>50/92a</td>
<td>9</td>
<td>–69 (7)*</td>
<td>2</td>
<td>39</td>
</tr>
<tr>
<td>Old DMPK+/–</td>
<td>49/102a</td>
<td>5</td>
<td>–70 (9)*</td>
<td>3</td>
<td>69</td>
</tr>
</tbody>
</table>

Multiple action potential was defined as two or more action potentials in response to a single stimulus. Wild-type, resting and action potentials were recorded from old muscle. There were no age related differences in membrane potential parameters in wild-type muscle (data not shown).

*P < 0.05 for the comparison with wild-type cells by ANOVA.
Effects of DMPK deficiency on skeletal muscle Na channels

Smaller Na currents in DMPK−/− muscle. Figure 1B shows representative families of macroscopic Na currents recorded from cell-attached patches in young (<30 weeks) wild-type and DMPK−/− muscle. The peak current amplitude was smaller in the patch from the DMPK−/− cell. Data from multiple patches confirmed this 50% reduction in amplitude (Fig. 1C, P < 0.05, multivariate rank sum test). The decay of macroscopic current was fit to a single exponential decay function, and the results in DMPK−/− muscle were not different from those in wild-type muscle (Fig. 1D), nor were there significant differences in equilibrium gating relationships in DMPK−/− muscle. Figure 1E shows that channel availability and conductance as a function of voltage were essentially identical in wild-type and DMPK−/− muscle.

Fewer Na channels DMPK−/− muscle. To determine the mechanism for the reduced peak current amplitudes in DMPK−/− muscle, we measured unitary Na currents in patches containing only a few channels. We specifically sought to determine whether the reduced peak current was due to a reduction in single channel amplitude, or a reduction in the number of channels firing simultaneously. Single channel amplitude at a test potential of 0 mV was unaffected by DMPK deficiency (0.95 ± 0.05 pA in wild-type muscle, compared with 1.05 ± 0.08 pA in DMPK−/− muscle). The number of channels per patch, assessed from the number of overlapping openings early after the onset of depolarization, on the other hand, decreased from 9.6 ± 0.9 in wild-type muscle to 5.2 ± 0.8 in DMPK−/− muscle (P < 0.001, Student's t-test), consistent with the 50% reduction in peak macroscopic current.

Na channel bursts and late plateau Na current in DMPK−/− muscle. The single channel experiments revealed a second effect of DMPK deficiency: late Na channel reopenings which summate to cause a plateau of non-inactivating current. Figure 2 shows representative sweeps and ensemble average currents from cell-attached patches containing a few channels in wild-type and DMPK−/− skeletal muscle isolated from young (<30 weeks), intermediate-age (30–60 weeks) and old (>60 weeks) mice. The expected finding is near-complete inactivation of Na currents within 5–10 ms, and no persistent current. This was the case for the patches from wild-type muscle, where bursts of channel activity in the first few milliseconds after depolarization were rarely followed by late channel opening (Fig. 2A). In wild-type muscle, non-inactivating current (i.e. persistent current at 30–40 ms after the step) accounted for only 0.52, 0.82 and 0.23% of peak ensemble averaged current in young (<30 weeks), intermediate-age (30–60 weeks) and old (>60 weeks) mice. The expected finding is near-complete inactivation of Na currents within 5–10 ms, and no persistent current. This was the case for the patches from wild-type muscle, where bursts of channel activity in the first few milliseconds after depolarization were rarely followed by late channel opening (Fig. 2A). In wild-type muscle, non-inactivating current (i.e. persistent current at 30–40 ms after the step) accounted for only 0.52, 0.82 and 0.23% of peak ensemble averaged current in young, intermediate-age and old mice, respectively (Fig. 2B). In DMPK−/− muscle, on the other hand, there were more Na channel openings and more long bursts, especially in older
mice (Fig. 2C). As a result, the ensemble average currents (Fig. 2D) displayed a larger non-inactivating pedestal of current. This effect was relatively small in the young DMPK –/– mice (1.6% of peak current) but was larger in intermediate-age (3.0%) and old (4.2%) mice.

**Increased Na channel bursting as DMPK –/– muscle aged.** To quantify the effect of DMPK deficiency on single channel gating, we constructed frequency histograms of NP\(_o\) from wild-type and DMPK –/– muscle. The histograms have been described with sums of two exponentials models (smooth lines). Note that there is a population of long bursts that is 3-5-fold higher in DMPK –/– muscle. The horizontal axis has a logarithmic scale with bin width 10^0.25. The data were derived from 10–24 patches and three to five mice for each condition.

**Figure 3.** (A–D) Frequency histograms of NP\(_o\) for wild-type (A) and DMPK –/– muscle from young (B), intermediate-age (C) and old (D) mice. Only the epoch from 10 to 110 ms after the beginning of the voltage step was analyzed. The left-most bar represents traces with no openings after 10 ms. The shaded area in each panel represents NP\(_o\) > 0.1. Reopenings were more frequent in Na channels from DMPK –/– muscle at all ages, and the defect was more marked as the mice aged. The horizontal axis has a logarithmic scale with bin width 10^0.25. The data were derived from 10–24 patches and three to five mice for each condition. (E and F) Burst duration histograms from old wild-type and DMPK –/– muscle. The histograms have been described with sums of two exponentials models (smooth lines). Note that there is a population of long bursts that is 3-5-fold higher in DMPK –/– muscle. The horizontal axis has a logarithmic scale, bin width 10^0.085. 

**Figure 3G–I** summarizes the gating properties of wild-type and DMPK –/– muscle at the three ages tested. We analyzed three parameters that were affected by DMPK deficiency. First, DMPK –/– muscle had more bursts of Na channel openings per channel (Fig. 3G), an effect that appeared to be more

**Figure 3J** Age effects on Na channel re-openings. P(NP\(_o\) > 0.1) is plotted as a function of age for 38 patches from wild-type (open circles) and 44 from DMPK –/– (filled circles) mice.
prominent in intermediate-age and old mice. Second, there were more long bursts of openings (Fig. 3H), and this effect appeared to be age independent. Third, DMPK–/– muscle exhibited more openings per channel (Fig. 3I), and this effect was also more apparent in older muscle. We used these three parameters as well as $P(N_{Po} > 0.1)$ to assess the significance of the difference between wild-type and DMPK–/– muscle, and the effect of increasing age, using a multivariate rank sum test. The difference between wild-type and DMPK–/– mice was significant at all ages ($P < 0.001$). In DMPK–/– mice, intermediate-age and old muscle were each significantly different from young muscle ($P < 0.05$) but not from each other. As described above, the increase in channel activity in DMPK–/– muscle was not the result of an increase in the number of Na channels per patch—in fact, this decreased.

Figure 3J shows a plot of $P(N_{Po} > 0.1)$ as a function of age for wild-type and DMPK+/– mice. The gating abnormality manifest as increased $P(N_{Po} > 0.1)$ was more evident in older DMPK+/– mice, and rarely present in wild-type mice. There was a modest but significant correlation for DMPK+/– muscle ($P < 0.02$, $r = 0.44$) but none for wild-type ($P = 0.25$).

**Similar Na channel gating abnormalities in DMPK+/– muscle.** The effect of partial DMPK deficiency was studied in intermediate-age muscle. In DMPK+/– muscle there was a reduction in current amplitude that was very similar to the reduction in amplitude observed in DMPK–/– muscle. Figure 4E shows current–voltage relationships for intermediate-age muscle. There was a significant reduction in current amplitude in both DMPK+/– and DMPK–/– muscle ($P < 0.05$, multivariate rank sum test), but no difference between the effect of partial and complete DMPK deficiency. As in DMPK–/– muscle, there were no significant shifts in equilibrium gating relationships with partial DMPK deficiency (data not shown).

Figure 4A shows representative single channel traces and ensemble average currents from intermediate-age wild-type and DMPK+/– muscle. As in DMPK–/– muscle, we found frequent late Na channel reopenings in DMPK+/– muscle. The ensemble average traces (Fig. 4B) show a plateau current in DMPK+/– muscle of 2.5% of peak, similar to the plateau current in intermediate-age DMPK–/– muscle. Figure 4C and D shows frequency histograms of $N_{Po}$. Data were derived from 10 patches from four mice for wild-type muscle, and nine patches from three mice for DMPK+/– muscle. Figure 4E shows pooled data for the peak current–voltage relationship in wild-type, DMPK–/– and DMPK+/– intermediate-age muscle. DMPK–/– and DMPK+/– muscle each had significantly smaller current amplitude than that of wild-type ($P < 0.05$ (35)), but were not different from each other. Data points are means ± SE from nine patches from three mice for each condition.
DISCUSSION

We studied skeletal muscle Na currents in genetically engineered mice with partial and complete DMPK deficiency. There were four important findings. (i) Muscle cells from DMPK-deficient mice were partially depolarized, and exhibited repetitive action potentials in response to single stimuli much more frequently than did wild-type muscle. (ii) Na channels in DMPK−/− muscle displayed more frequent and longer openings, and longer bursts of openings during sustained depolarization. This recapitulates the Na channel abnormality of human DM (25), and provides a link between DMPK deficiency and altered Na channel gating. (iii) The Na channel lesion was identical in muscle heterozygous and homozygous for DMPK loss, indicating that partial deficiency of DMPK is sufficient to reproduce the Na channel disease phenotype. (iv) The Na channel lesion was much more prominent with increasing age, compatible with the progressive nature of the myopathy seen in the intact animal (21), and also with the human disease. These findings bring up two important questions. First, does the Na channel gating abnormality help to explain progressive skeletal muscle weakness, the major phenotype of DMPK-deficient mice? Second, could the Na channel lesion, in conjunction with other lesions of CTG expansion contribute to myotonia?

Our data do not address the mechanism by which DMPK might affect Na channels. Although a direct demonstration of DMPK phosphorylation of Na channels has not yet been reported, our previous studies of muscle Na channels co-expressed with DMPK in Xenopus oocytes (26) indicated that the mechanism was likely to involve phosphorylation of the Na channel molecule. We demonstrated that the effects of DMPK co-expression on Xenopus oocyte-expressed Na channels were absent in a Na channel mutant in which a phosphorylation site had been disabled by Ser→Ala mutation. These data are consistent with the idea that phosphorylation of the Na channel is required in the response of the channels to DMPK, a serine/threonine kinase.

It would be of interest to measure DMPK levels in DMPK−/− muscle in an attempt to correlate DMPK levels with the Na channel abnormalities. Although DMPK mRNA levels are reduced by 50%, DMPK protein and its kinase activity may also be regulated at the post-transcriptional or post-translational level. Assessment of DMPK levels in murine muscle awaits development of an antibody that reliably recognizes murine DMPK.

Our data show an increasing degree of abnormality of Na channel gating in older mice. This finding is consistent with not only the progressive nature of the muscle weakness in DM patients, but also with the prior studies of muscle function in DMPK−/− mice (21). Specifically, Reddy et al. (21) found no decrease in muscle force and tetanic force generation in 3- to 4-month-old DMPK−/− mice, but a 30–50% reduction in 7- to 11-month-old mice. Thus, our study of Na channel gating is consistent with the age-related deterioration of the human disease as well as the genetically engineered mouse physiology.

The mechanism of the skeletal muscle weakness has been suggested to be an abnormality of excitation–contraction coupling, as in vitro studies of myotubes from DMPK−/− mice show a 40% reduction of Ca release from the sarcoplasmic reticulum in response to depolarization with acetyl choline or KCl (22). A role for Na channels was suggested because the specific Na channel blocker, tetrodotoxin, improved both the amplitude and kinetics of the Ca transient. Persistent membrane depolarization in DMPK-deficient muscle would inhibit complete recovery from inactivation of the Ca channels, reducing the magnitude of Ca current after any depolarizing stimulus. This would reduce Ca release from the sarcoplasmic reticulum, and hence also contraction. The progressive nature of the Na channel abnormality is consistent with the progressive abnormalities of excitation–contraction coupling and of skeletal muscle weakness. There are other possibilities, however. First, the Na channel β-subunit is a substrate for phosphorylation by DMPK (29), allowing the possibility of modulation of Ca channels by DMPK. Second, the sarcoplasmic Ca ATPase is deficient in human DM (30,31) and this too may contribute to skeletal muscle weakness.

DM patients possess one wild-type chromosome and one mutant chromosome. As the CTG tract progressively expands in size, DMPK levels are predicted to drop from 100 to 50% of wild-type levels. As loss of one allele in most genes is usually insufficient to produce pathology, it is unclear why the wild-type DMPK allele is unable to maintain the normal phenotype. There are two possible mechanisms. First, when DMPK levels fall below a relatively sharp threshold, an all-or-none effect may be observed in one or more DMPK targets; that is, below a threshold, a target molecule would be insufficiently phosphorylated and show functional abnormalities. In this model, both heterozygous and homozygous mutant animals would show a similar phenotype if the DMPK threshold level required for normal function is near to 50% of the wild-type level. Alternatively, changes in DMPK levels may show a graded response; that is, decrements in DMPK levels would result in a corresponding decrease in the number of a particular phosphorylated target. In this model, the phenotype exhibited by the heterozygous mutant animals would be intermediate to that observed in wild-type and homozygous mutant animals. These models may be tested using a physiological function that relates directly to the phosphorylation of a single DMPK target, as opposed to complex phenomena, such as muscle contraction, that are predicted to result from a composite of several direct and indirect DMPK targets. As previous studies have suggested that Na channels may be phosphorylated as a result of DMPK activity (26,27), and we have attempted to distinguish the two models by studying the effect of DMPK dosage on Na channel function in vivo. Our results support a model where the relevant Na channel defect occurs in an all-or-none fashion as a function of DMPK dosage. Thus, these results provide a viable molecular explanation as to why relatively small alterations in DMPK levels could result in large phenotypic differences in DM patients (17).

However, Reddy et al. (21) reported significant variability in skeletal muscle force production in animals heterozygous for DMPK deficiency with only two of six DMPK−/− mice showing substantial decreases in twitch and tetanic force development. Since we would expect that all six DMPK−/− muscles would have abnormal Na channel gating, we conclude that the abnormalities of excitation–contraction coupling in DMPK deficient muscle are multifactorial. For example, the phosphorylation of the Ca channel β-subunit by DMPK may not occur in a similar all-or-none fashion.

Why were the effects of DMPK deficiency on Na channel gating enhanced with increasing age? Age-related changes in
skeletal muscle Na channel gating have been reported in rat skeletal muscle (32), and possible mechanisms include changes in the subunit composition of the channel, channel glycosylation and/or phosphorylation. These may possibly enhance the sensitivity of the channel to alterations in DMPK dosage.

Could the Na channel lesion we report here contribute to myotonia in DM? DMPK-deficient mice do not exhibit the robust myotonia characteristic of the human disease. We demonstrate, however, that DMPK-deficient muscle exhibits multiple action potentials in response to single stimuli, whereas wild-type muscle does not. We consider that this is most likely a result of the Na channel gating abnormality because the repeated action potentials were blocked by lidocaine. It is of interest that our data recapitulate Na current and membrane potential data of Franke et al. (25) from isolated human DM muscle. Our data suggest that the Na channel abnormality may contribute to muscle hyperexcitability in conjunction with other molecular defects resulting from CTG expansion.

In conclusion, our results link DMPK activity to skeletal muscle Na channel function. Increased Na current resulting from late Na channel openings in DMPK-deficient mice may contribute to the weakness characteristic of DM, and, in conjunction with other molecular defects, possibly also to the myotonia. More studies of mouse models engineered to recapitulate molecular disease mechanisms, though, should continue unravel this fascinating and prismatic illness.

MATERIALS AND METHODS

Mice deficient in DMPK

The DMPK–/– 129SV mouse model that we studied has been described previously (21). Homozygous DMPK–/– and heterozygous DMPK+/– mice were studied over a range of ages. Young mice were up to 30 weeks old; intermediate-age mice were 30–60 weeks old; and old mice were >60 weeks old.

Isolation of mouse skeletal muscle cells

Mice were anesthetized with intraperitoneal pentobarbital (0.033 mg/g) and sacrificed by cervical dislocation using a protocol approved by the University of Virginia Animal Care Committee. The hind limb was excised above the knee joint and transferred to oxygenated Tyrode’s solution at 37°C for 90 min. Tyrode’s solution contained 120 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2.2 mM CaCl2, 0.5 mM NaH2PO4, 10 mM HEPES and 11 mM glucose pH 7.4 (NaOH). The flexor digitorum brevis was isolated complete with tendons. The number of channels was estimated from the number of overlapping openings at strong depolarizations. Late openings during a 100 ms period beginning 10 ms after the voltage step were analyzed. Bursts were identified using a critical closed time of 5 ms. Dwell time histograms were described with sums of exponentials models using a maximum likelihood technique (33).

Electrophysiological recording

Membrane potential recordings and cell-attached patch recordings were made at room temperature (20°C) by standard techniques using an Axopatch 200A (Axon Instruments, Foster City, CA) amplifier and pCLAMP (Axon Instruments) hardware and software. Membrane potential recordings were made with standard 3 M KCl-filled microelectrodes. Macroscopic currents were filtered at 2 kHz and sampled at 66 kHz; single channel recordings were filtered at 2 kHz and sampled at 10 kHz. For macroscopic recordings, electrode resistance was 3–5 MΩ; for single channel recording 10–12 MΩ electrodes were used. The electrodes were filled with the enzyme-free Tyrode’s solution containing also 2 mM BaCl2, 2 mM CdCl2, 5 mM TEA, 5 mM 4-AP and 2 mM 9-AC to block Ca, K and Cl currents. The patch was held at −120 mV relative to the membrane potential, which was held near to 0 mV by the depolarizing K-aspartate solution. Currents were analyzed using pCLAMP (Axon Instruments), Transit (34) and Origin (MicroCal, Northampton, MA).

The decay phases of macroscopic currents were fit to a single exponential decay function (Microsoft Excel). Conductance as a function of voltage was derived from the peak current–voltage relationship. Channel availability as a function of voltage were determined using a two-pulse protocol, with a 100 ms step from the holding potential (−120 mV) to the test potential at 0.2 Hz. Currents were measured at −10 mV. The raw data for each determination were fit to Boltzmann functions (Microsoft Excel).

The number of channels was estimated from the number of overlapping openings at strong depolarizations. Late openings during a 100 ms period beginning 10 ms after the voltage step were analyzed. Bursts were identified using a critical closed time of 5 ms. Dwell time histograms were described with sums of exponentials models using a maximum likelihood technique (33).

Statistical analysis

For comparison of the incidence of action potential bursts, and for comparisons of resting membrane potential we used a Kruskal–Wallis one-way ANOVA on ranks because the data were not normally distributed. For multiple comparisons between the groups, Dunn’s method was used.

For several analyses we used a non-parametric multivariate rank sum test (34). For the current–voltage relationship data shown in Figures 1C and 4E, we used one-way ANOVA on the sums of ranks of the current amplitudes at the 15 test potentials. We used a Tukey test for pairwise comparison for the data in Figure 4E. To test the hypothesis that complete DMPK deficiency altered Na channel gating, we measured four properties of single channels in two types of mouse at three ages and extended the technique to two-way ANOVA to yield an approximation of the P-value, with a Tukey test for pairwise comparisons. We used one-way ANOVA to test the
significance of effect of gene dose on the same parameters of channel gating at one age.

The significance of the correlation between gating and age shown in Figure 3J were tested by calculating the non-parametric Spearman rank correlation coefficient (SigmaStat, Jandel). \( P < 0.05 \) was considered statistically significant. Data are given as mean \( \pm \) SE.

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