Sodium Currents in Mesencephalic Trigeminal Neurons From Na_v1.6 Null Mice

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Enomoto A, Han JM, Hsiao C-F, Chandler SH. Sodium currents in mesencephalic trigeminal neurons from Na_v1.6 null mice. J Neurophysiol 98: 710–719, 2007. First published May 23, 2007; doi:10.1152/jn.00292.2007. Previous studies using pharmacological methods suggest that subthreshold sodium currents are critical for rhythmic burst generation in mesencephalic trigeminal neurons (Mes V). In this study, we characterized transient (I_{NaT}), persistent (I_{NaP}), and resurgent (I_{res}) sodium currents in Na_v1.6-null mice (med mouse, Na_v1.6<sup>−/−</sup>) lacking expression of the sodium channel gene Scn8a. We found that peak transient, persistent, and resurgent sodium currents from med (Na_v1.6<sup>−/−</sup>) mice were reduced by 18, 39, and 76% relative to their wild-type (Na_v1.6<sup>+/+</sup>) littermates, respectively. Current clamp recordings indicated that, in response to sinusoidal constant amplitude current (ZAP function), all neurons exhibited membrane resonance. However, Mes V neurons from med mice had reduced peak amplitudes in the impedance-frequency relationship (resonant Q-value) and attenuated subthreshold oscillations despite similar passive membrane properties compared with wild-type littermates. The spike frequency-current relationship exhibited reduced instantaneous discharge frequencies and spike block at low stimulus currents and seldom showed maintained spike discharge throughout the stimulus in the majority of med neurons compared with wild-type neurons. Importantly, med neurons never exhibited maintained stimulus-induced rhythmic burst discharge unlike those of wild-type littermates. The data showed that subthreshold sodium currents are critical determinants of Mes V electrogenesis and burst generation and suggest a role for resurgent sodium currents in control of spike discharge.

INTRODUCTION

Voltage-dependent sodium channels are critically important for production of the action potential and spike propagation in neurons, and at the molecular level, a number of sodium channel isoforms have been identified and their electrical properties characterized (Catterall 2000). In addition to the fast, transient sodium currents responsible for spike generation, slowly or noninactivating sodium currents, termed persistent sodium currents (I_{NaP}), have been identified and shown to participate in control of subthreshold membrane excitability and repetitive firing characteristics (Crill 1996; Del Negro et al. 2002; Taylor 1993; van Drongelen et al. 2006; Wu et al. 2001, among others). More recently, some neurons were found to exhibit an additional current called resurgent sodium current (I_{res}) (Raman and Bean 1997) that occurs during action potential repolarization and is associated with rapid recovery from sodium channel inactivation. These properties suggest that this current contributes to high-frequency spike discharge. In Purkinje neurons, I_{res} is associated predominately with the presence of the Na_v1.6 isoform (Raman et al. 1997), but that is not true for all types of neurons (Do and Bean 2003). A major goal is to associate the underlying sodium channel isoforms with unique electrical properties of the neuron. This will provide us with important information regarding the roles of these isoforms in normal and pathological electrogenesis.

Our previous in vitro studies showed that mesencephalic trigeminal (Mes V) neurons, critical components of circuits controlling oral-motor activity in the brain stem, exhibit membrane resonance, voltage-dependent subthreshold oscillations, and rhythmic burst behavior on membrane depolarization (Wu et al. 2001, 2005). The membrane resonance is dependent on a low threshold, 4-aminoopyridine (4-AP) sensitive potassium current and is amplified by I_{NaP} to produce subthreshold oscillations and bursting. A subsequent in vitro study showed that during a given burst, both I_{NaP} and I_{res} flow at different times during the burst cycle (Enomoto et al. 2006). Therefore to directly test the contribution of these sodium current components to the genesis of Mes V excitability and bursting, we examined these currents and subsequent membrane excitability in mice that are homozygous for a null allele of Na_v 1.6 (med mouse) (Burgess et al. 1995) that in Purkinje neurons reduces I_{NaP} and I_{res} substantially (Raman and Bean 1997). These mice show altered motor functions, such as ataxia and progressive paralysis, before death, which occurs around 3 wk of age (Meisler et al. 2001).

In this study, we found that, indeed, in med mice neurons, I_{NaP} and I_{res} are significantly reduced, and spike frequency and discharge characteristics are altered both qualitatively and quantitatively, indicating that these currents play an important role in regulating Mes V neuronal excitability and discharge characteristics.

METHODS

Heterozygous Scn8a<sup>med</sup> mice maintained in strain C3Heb/FeJ were obtained from Jackson Laboratories (Bar Harbor, ME). The med mutation produces complete loss of Na_v1.6 expression. For the initial experiments, genotyping was performed after the experiments. However, for most experiments to compare only homozygous null animals with wild-type animals and increase the efficiency of our experiments, genotyping was performed for mice before experiments, and we selected only homozygous med (Na_v1.6<sup>−/−</sup>) or wild-type (Na_v1.6<sup>+/+</sup>) littermates.

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After extracts from mouse tails were obtained, the tails were incubated in 0.4 ml of lysis buffer (100 mM NaCl, 10 mM Tris, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml Proteinase K) in a 50°C waterbath overnight. After the samples were centrifuged at 6,000 rpm for 10 min, the supernatant was transferred to a tube containing 400 µl of isopropanol. The DNA was collected using flame-sealed capillary pipette and was dissolved in Tris-EDTA buffer, pH 7.6. PCR amplification used the following primers (5 ′ to 3 ′): for the wild-type allele, GGAGCAGGTGTCTAGGCGAGTTTAAGTGTG and GTGCAAGGGCAGCTGTGCACACCT ACCATTC (Kohman et al. 1996), and for the mutant allele, TCCAAAGCTTATAACAAAAGTCCC and GGAGCAGGCACTCATCTAGTCC (Integrated DNA Technologies). The reaction consisted of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C (30 repetitions), and 5 min at 72°C. PCR products were separated on a 2% agarose gel, allowing resolution of a 230-bp product for the wild-type allele and a 194-bp product for the mutant allele.

Slice preparation

Experiments were performed on Mes V neurons. Coronal slices from neonatal mice (postnatal 8–14 days) were cut by the protocol previously described (Wu et al. 2001). Briefly, animals were rapidly decapitated, and the brains were quickly removed and immersed in artificial cerebrospinal fluid (ACSF) (Wu et al. 2001; Zhang and Krnjevic 1993). This was produced by sequentially immersing a patch electrode in the electrode solutions followed by artificial cerebrospinal fluid (ACSF) (Wu et al. 2001). The mesencephalic trigeminal nucleus was identified with oxygenated ACSF (2 ml/min) at room temperature, and visualized solution. Slices were secured in a recording chamber, perfused at 60–90%, and monitored periodically throughout the experiment. The liquid junction potential was usually 3–5 mV when filled with the intracellular solution. Slices were secured in a recording chamber, perfused with oxygenated ACSF (2 ml/min) at room temperature, and visualized by infrared differential interference contrast microscopy (Stuart et al. 1993). The mesencephalic trigeminal nucleus was identified bilaterally in the coronal slice under low magnification (×5) as an ellipsoid region, which is located dorsally in brain stem slices ~500 µm lateral to the midline. Mes V neurons were easily distinguished based on their location, pseudounipolar soma, and size (Del Negro and Chandler 1997; Henderson et al. 1982). The effects of drugs applied to the bath solution were obtained after 3–10 min of application. Recording periods were usually between 30 and 60 min.

Current/voltage-clamp experiments

For current-clamp experiments, borosilicate pipettes were filled with an internal solution containing (in mM) the following composition: 115 K-glucocan, 25 KCl, 9 NaCl, 10 HEPES, 0.2 EGTA, 1 MgCl2, 3 K3-ATP, and 1 Na-GTP. The pH was adjusted to 7.25 with KOH, osmolarity adjusted to 280–290 mosmol. The control external solution consisted of ACSF of the following composition (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 26 NaHCO3, 10 glucose, 2 CaCl2, and 2 MgCl2.

Voltage-clamp experiments were designed to isolate sodium currents and block potassium currents. For those experiments, the internal solution was composed of the following (in mM): 130 CsF, 9 NaCl, 10 HEPES, 10 EGTA, 1 MgCl2, 3 K3-ATP, and 1 Na-GTP. The external solution contained the following (in mM): 131 NaCl, 10 HEPES, 3 KCl, 10 glucose, 1 CaCl2, 2 MgCl2, 10 tetraethylammonium (TEA)-Cl, 10 CsCl, 4-AP, and 0.3 CaCl2. Sodium currents were defined by subtracting the currents remaining in 0.5 µM TTX. In some experiments, to measure the transient sodium current, the external Na+ concentration was reduced to 50 mM and substituted with 91 mM TEA to minimize series resistance resistance errors.

Acquisition and analysis

Currents and voltages were digitized and controlled by pClamp 9.2 software (Axon Instruments). Data were collected and analyzed with a combination of software [Clampfit (ver 9.2, Axon Instruments), StatView (SAS Institute, Cary, NC), and Microsoft Excel]. Traces shown in some figures were digitally filtered with an effective corner frequency of 3 kHz (8-pole Bessel filter).

In neurons that showed burst discharge, the mean peak-to-peak amplitude of subthreshold oscillations occurring between bursts at different membrane potentials was determined by averaging the peak amplitude of oscillation within five equal intervals of time between subsequent burst discharges. We obtained the mean oscillation peak amplitude for three periods lasting 1–2 s at each voltage level. The peak subthreshold oscillation frequency was determined from fast Fourier transform (FFT) analysis by measuring the voltage region between two subsequent burst discharges. In the absence of burst discharge, FFT was constructed from epochs of 1- to 2-s duration. We averaged the results from three different epochs, plotted the power-frequency relationship, and obtained peak frequencies.

Frequency-domain analysis (Puil et al. 1986, 1988; Wu et al. 2001) was performed by injecting a computer-generated impedance amplitude profile (ZAP) input current of changing frequencies between 0 and 250 Hz into neurons and recording the resulting voltage responses. To carefully analyze the subthreshold membrane properties in the absence of spikes, the amplitude of the ZAP input function was adjusted to keep the peak-to-peak voltage responses <10 mV. The current and voltage records were digitized at frequencies of 10 kHz. Impedance (Z) was calculated from the ratio of the FFT of the voltage response and the input current using the formula: Z = FFT(V)/FFT(I). The magnitude of the impedance was plotted against frequency to give a frequency-response curve (FRC). Once the FRC was obtained, the resonant behavior, if present, was quantified by measuring the resonant frequency (Fres) and the Q value. The Fres was defined as the frequency at the peak of the hump in the FRC. The Q value was calculated by measuring the impedance at Fres and dividing that by the magnitude of the impedance at the lowest frequency measured (usually 1 Hz) (Hutcheon et al. 1996; Koch 1984). A Q value of 1 would indicate that there was no resonance present, whereas values >1 indicate some degree of resonant behavior and therefore a particular frequency preference for the neuron. ZAP input current was generated with the formula: I(t) = a sin (bt2), 0 ≤ t ≤ T. Here, a and b are adjustable parameters controlling the amplitude and bandwidth
of the input current, respectively. \( T \) was a finite duration. In our case, \( a = 5, b = 10^{-7}, t = 8 \) s, and \( T = 10 \) s. The frequency applied was between 0 and 250 Hz. We used a low-pass filter of 0.5 kHz to reduce the noise of the input current and voltage. The results with and without the low-pass filter were identical.

Results are reported as mean ± SD, unless indicated otherwise. The group comparison of mean values was performed with a Mann-Whitney \( U \) test at a level of significance of \( P < 0.05 \) unless otherwise stated.

**RESULTS**

**Transient sodium current**

Initially, we compared transient sodium current properties in Mes V neurons of wild-type (wild-type; \( \text{Na}_1.6^{+/+} \) and med (\( \text{Na}_1.6^{-/-} \)) mice using standard step voltage protocols. Figure 1A (top) shows a family of fast transient inward currents evoked by depolarization to potentials between \(-80 \) and \(-15 \) mV from a holding potential of \(-70 \) mV for both wild-type (top left) and med mice (top right). The external solution contained reduced (15 mM) sodium to minimize series resistance errors and obtain good voltage control (Enomoto et al. 2006). There was no significant difference in the voltage dependence of the peak transient current in cells from wild-type and med mice. In both cases, peak transient current increased steeply from \(-50 \) to \(-30 \) mV, and maximal current was elicited by steps to around \(-25 \) mV. As shown in Fig. 1B, the magnitude of the mean peak current was smaller in cells from med mice than from wild-type. When the absolute peak currents were normalized for differences in cell size using cell capacitance as an indicator, the current density for maximal currents were normalized for differences in cell size using cell capacitance as an indicator, the current density for maximal current was reduced by 18% in cells from med mice \((-21.1 \pm 10.3 \) pA/pF, \( n = 8 \)) compared with that of wild-type \((-25.8 \pm 11.5 \) pA/pF, \( n = 8 \)). However, this difference was not significant.

The peak current–voltage relationships were similar between both wild-type and med mice. The voltage dependence of activation was not significantly different in wild-type and med mice when measured quantitatively by conductance–voltage plots (Fig. 1C). The data for each cell were fit with a Boltzmann function, \( 1/[1 + \exp(-(V - V_h)/k)] \), where \( V \) is the test potential. \( V_h \) is the midpoint, and \( k \) is the slope factor in millivolts. The midpoint for wild-type was \(-32.9 \pm 0.2 \) mV, whereas Med mice exhibited a 1.6-mV shift in the depolarizing direction \((V_h = -31.3 \pm 0.4 \) mV). Slope factors were similar in neurons from wild-type \((k = 6.1 \pm 0.2) \) and med mice \((k = 6.2 \pm 0.3) \). Although there was a small shift to the depolarizing voltage, the voltage dependence of activation was not significantly different between two groups.

The voltage dependence for inactivation was measured by applying 100-ms prepulses between \(-120 \) and \(+10 \) mV and determining availability of sodium channels with a test step pulse to \(-10 \) mV (Fig. 1A protocol, bottom right). A family of step pulses and the subsequent currents for wild-type (left bottom) and med mice (bottom right) are shown. Data were fit well by the Boltzmann function \( 1/[1 + \exp((V - V_h)/k)] \). The properties of inactivation also showed no significant differences between wild-type \((V_h = -61.9 \pm 0.5, k = 9.5 \pm 0.4, n = 5) \) and med mice \((V_h = -61.2 \pm 0.7, k = 9.4 \pm 0.6, n = 5) \). Furthermore, the time constant for fast and slow inactivation at \(-30 \) mV for both populations was similar.

\( \text{FIG. 1. Transient sodium current properties of wild-type and med mice neurons. A: transient sodium current traces evoked by activation (top, left and right) and inactivation (bottom, left and right) protocols in low extracellular sodium solution (15 mM). Duration of conditioning pulse was 100 ms during inactivation protocol (protocols are shown as insets). B: composite peak current-voltage relationship for sodium currents obtained from med mice (○) and wild-type neurons (●). C: composite conductance-voltage relationship of sodium currents obtained from med mice (○) and wild-type neurons (●). Conductance (G) was calculated as G = I/(V - \( E_{\text{rev}} \)), normalized to the maximal conductance (\( G_{\text{max}} \)), and plotted as a function of step depolarization. Reversal potential (\( E_{\text{rev}} \)) of peak transient currents was +10.4 mV. Dotted line is Boltzmann fit for med mice data and solid line is fit to wild-type data.} \)

\( \text{(WT; } \tau_{\text{fast}} = 0.75 \pm 0.24 \) ms, \( \tau_{\text{slow}} = 3.60 \pm 1.1 \) ms; \( \text{med, } \tau_{\text{fast}} = 0.65 \pm 0.11 \) ms, \( \tau_{\text{slow}} = 3.58 \pm 1.22 \) ms, \( n = 8, P > 0.05 \)).

**Persistent sodium current**

To characterize persistent and resurgent sodium current components, the following protocols were used (Do and Bean...
Resurgent current was measured at −40 mV after a brief step to +30 mV. The amplitude of $I_{\text{res}}$ was calculated as the peak current minus the amplitude of the current remaining at the end of the pulse (persistent Na⁺ current; Fig. 2A, left). As shown previously in Mes V neurons (Enomoto et al. 2006), this protocol maximally activates resurgent current within this voltage window. Initially, persistent current was measured at the end of the repolarizing steps. The 100-ms pulse was short enough to measure persistent Na⁺ current in the absence of any slow inactivation. However, to more rapidly measure $I_{\text{res}}$ over an entire voltage range and generate an I-V relationship, slow ramp voltage commands that spanned −90 to +10 mV were subsequently used (33.3 mV/s; Fig. 2A). During these conditions, the ramp and steady-state currents measured were the same as shown for the example in Fig. 2A (dashed horizontal line) taken at −40 mV. The ramp protocol speed was sufficient to completely inactivate the transient Na⁺ current yet allow maintenance of the slowly or noninactivating persistent component.

In Mes V neurons from wild-type mouse, persistent sodium current activated around −75.0 ± 5.5 mV and showed a peak current at −46.4 ± 3.8 mV ($n = 8$), whereas in $med$ mice, persistent sodium current activated around −72.2 ± 3.7 mV and exhibited a peak current at −45.1 ± 4.4 mV ($n = 10$). This difference was not significant. Examples of ramp data taken from a wild-type and $med$ neuron are shown in Fig. 2B. When the absolute currents were normalized for differences in cell size as indicated by changes in cell capacitance, the current density of maximal peak amplitude induced by the ramp protocol was reduced by 39% in $med$ mice neurons ($−1.6 ± 0.5$ pA/pF; $n = 10$) compared with wild-type ($−2.6 ± 0.7$ pA/pF; $n = 8$; $P < 0.01$).

Although the peak current–voltage relationships were similar between both wild-type and $med$ mice, the voltage dependence of activation was significantly different in wild-type and $med$ mice when conductance–voltage plots were examined (Fig. 2C). The data for each cell (wild-type $n = 5$, $med$ $n = 8$) were fit with a Boltzmann function, $1/(1 + \exp[-(V - V_m)/k])$, where $V$ is the test potential, $V_m$ is the midpoint, and $k$ is the slope factor in millivolts. The midpoint for wild-type was −61.2 ± 2.1 mV. $Med$ mouse showed a 4.2-mV shift in the depolarizing direction, with $V_m = −57.0 ± 2.9$ mV ($P < 0.05$). Slope factors were similar in neurons from wild-type ($k = 5.3 ± 0.4$) and $med$ mice ($k = 4.9 ± 1.0$).

**Resurgent sodium current**

Resurgent sodium current was present in Mes V neurons from both wild-type and $med$ mice and was similar to that found previously in rat Mes V neurons (Enomoto et al. 2006). Figure 3A shows a typical example of the currents taken from a wild-type (Fig. 3A, top) and $med$ mouse (Fig. 3A, bottom). The most striking difference was in the reduced amplitude of the peak $I_{\text{res}}$ in $med$ mice. Figure 3B (top) shows the summary I-V relationship for the peak amplitude of $I_{\text{res}}$. As seen, the peak amplitudes occurred at approximately −40 mV for both groups. In addition to differences in total peak current, the current densities where reduced ~76% in $med$ mice compared with wild-type mice ($med$, 3.0 ± 0.9 pA/pF; $n = 15$ vs. wild-type, 12.5 ± 6.2 pA/pF; $n = 10$; $P < 0.01$; Fig. 4).

In addition to changes in amplitudes of $I_{\text{res}}$, the kinetics of this current between the two groups varied more modestly. When neurons were repolarized from +30 to −40 mV, where $I_{\text{res}}$ is maximal, the $I_{\text{res}}$ in $med$ neurons reached a peak in 7.9 ± 1.1 ms ($n = 15$), which was statistically later than that observed in wild-type neurons (3.8 ± 1.1 ms, $n = 10$; $P < 0.01$; Fig. 3B, middle). However, the resurgent current at −40 mV decayed with a time constant (tau) of 24.1 ± 6.2 ms in $med$ neurons, which was similar to that observed in wild-type neurons (23.8 ± 7.5 ms; Fig. 3B, bottom). Figure 4 summarizes the differences between wild-type and $med$ mice for all three components of the sodium current measured at −40 mV.

**Membrane properties and rhythmical burst generation**

Table 1 shows the values of some basic passive membrane properties for both wild-type and $med$ mice neurons. Resting membrane potential differed, significantly, by −2 mV,
mV that in a subset of neurons (produced subthreshold, voltage-dependent oscillations of 1–5 mV after maximal fast inactivation occurred. Regardless of whether bursting was initiated, wild-type mouse neurons always exhibited subthreshold oscillations, as previously shown in rat Mes V neurons (Wu et al. 2001). However, in the med mice population, bursting could not be induced (n = 0/44). Figure 5A shows a typical example of the membrane potential response to maintained current injection. Most often only a single spike occurred at the onset of the current pulse. Concomitant with this, the subthreshold oscillations were present and voltage-dependent, but of very small amplitude (Fig. 5B; Table 2), and peaks in the FFT were very small. The power in the peak frequency of the FFT from the med mice neurons was significantly smaller (med mice, 0.008 ± 0.001 mV2/Hz, n = 15 vs. wild-type, 0.015 ± 0.002 mV2/Hz, n = 18; P < 0.01) compared with wild-type neurons. As predicted (see DISCUSSION), the frequency of the oscillations was not significantly different between the two groups. This further supports the hypothesis previously put forward (Wu et al. 2001, 2005) that I_NaP is important for amplification of the subthreshold oscillations but does not directly regulate subthreshold membrane potential oscillation frequency.

Frequency domain analysis: impedance measurements

To more carefully examine the changes in subthreshold membrane properties that occur between wild-type and med mice, we performed frequency domain analysis on the membrane potential (see METHODS) (Hutcheon and Yarom 2000; Puil et al. 1986; Wu et al. 2001). Figure 6A shows an example of a ZAP input current and subsequent voltage output from a wild-type and med mouse neuron at −46 mV, whereas Fig. 6B shows the FRC (see METHODS) constructed from such data. In

### Table 1. Membrane properties of Mes V neurons

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<tr>
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<th>Resting Membrane Potential, mV</th>
<th>Input Resistance, MΩ</th>
<th>Cell Capacitance, pF</th>
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<tr>
<td>Wild-type</td>
<td>−65.4 ± 4.0 (25)</td>
<td>123.0 ± 57.6 (33)</td>
<td>72.9 ± 22.0 (40)</td>
</tr>
<tr>
<td>Mutant</td>
<td>−63.4 ± 3.9 (55)*</td>
<td>131.1 ± 66.8 (57)</td>
<td>58.9 ± 17.7 (69)†</td>
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Values are means ± SD with number of cells in parentheses for each group. Averaged group values were compared with Student’s t-test; *P < 0.05. †P < 0.01. Mes V, mesencephalic trigeminal.
all wild-type and med mice neurons examined, when the membrane potential was depolarized, the $Q$ value increased, the $F_{res}$ shifted to higher values, and the width of the FRC narrowed, reflecting voltage dependence for the impedance, as previously shown in rat Mes V neurons (Wu et al. 2001). When measured at holding potentials between $-66$ and $-46$ mV, all of the neurons showed a single resonant peak between 20 and 135 Hz. In contrast to that observed for wild-type Mes V neurons, med mice neurons showed a significant reduction in impedance peak amplitude and $Q$ value, reflecting a lesser degree of membrane resonance. Table 2 summarizes some of the characteristics of resonance for Mes V neurons measured at $-46$ mV for wild-type and med mice. Previously, we suggested that $I_{NaP}$ is primarily responsible for the magnitude of the resonant $Q$ value; reduction of $I_{NaP}$ with low doses of TTX sufficient to suppress $I_{NaP}$ significantly reduced the $Q$ value and the amplitude of the subthreshold oscillation (Wu et al. 2001). This was further supported with a computational model (Wu et al. 2005). The data obtained using the med mouse more directly links the role of Nav1.6 and $I_{NaP}$ to control of the amplitude of the membrane resonance and subthreshold oscillation.

**Frequency-current relationship and discharge patterns**

From the above experiments, it is clear that the ability to induce rhythmical burst discharge in med mice is significantly compromised. Recently, in an elegant study, it was shown experimentally and computationally that $I_{res}$ contributes to control of discharge frequency in cerebellar Purkinje neurons (Khaliq et al. 2003). Because $I_{res}$ is significantly reduced in Mes V neurons from med mice and previously we showed that $I_{res}$ does flow during spike repolarization and the early part of the interstimulus interval (ISI) (Enomoto et al. 2006), we...
sought to more carefully examine differences in spike frequency, discharge pattern, and membrane excitability in the two groups of animals. Figure 7 shows a typical example of the spike discharge response to a 1-s depolarizing current pulse from a wild-type and med mouse neuron. Spike discharge was obtained in response to a current pulse that produced maximal discharge frequency just before the onset of spike block. At a holding potential of −50 mV, which inactivates transient potassium currents (Del Negro and Chandler 1997), 10/16 wild-type neurons showed maintained discharge throughout the current pulse elicited at rheobase (Fig. 7A). These neurons were classified as tonic discharge neurons (T). In the remaining six wild-type neurons, a short burst of spikes occurred at rheobase that was followed by complete spike cessation and were classified as phasic discharge neurons (P; data not shown). Further increases in intensity did not induce tonic discharge in these neurons. In contrast, phasic discharge at rheobase was most often observed in med mice neurons (12/23; Fig. 7A, mutant), whereas tonic discharge was less frequently seen. Additionally, in med neurons, single spike discharge (S type neuron) in response to maximal current stimulation was frequently observed. Single spike discharge was never observed in wild-type neurons. The distribution of neurons according to firing pattern is shown in Fig. 7B. For those neurons that exhibited either phasic or tonic discharge, the maximal first ISI in response to a suprathreshold 1-s stimulus pulse of maximal intensity (intensity set to just below the onset of depolarization block) was 18% lower for med neurons (138.2 ± 15.6, n = 13) compared with wild-type (168.9 ± 26.0 n = 13; unpaired Students t-test, P < 0.001). The difference between wild-type and med neurons is more readily apparent when the number of spikes in response to a 1-s stimulus is plotted versus stimulus intensity for all neurons examined. As shown, wild-type neurons produced greater numbers of spikes prior to spike block compared with med neurons at all intensities examined. The number of spikes elicited at 600 pA for med neurons (6.8 ± 3.5, n = 23) was significantly lower compared with wild-type (20.5 ± 5.5, n = 16, P = 0.05, unpaired Students t-test). Furthermore, the onset of spike block in med neurons occurred at lower intensities compared with wild-type neurons.

Mes V neurons have a strong voltage-dependent steady-state low threshold 4-AP sensitive K⁺ current, which is responsible for the strong adaptation observed in most of these neurons during current pulses (Del Negro and Chandler 1997; Wu et al. 2001). To examine the steady-state discharge and obtain additional information on the role of the sodium current components in the absence of the strong membrane shunt produced by the 4-AP sensitive current, in some experiments, a low dose of 4-AP (50 μM) was applied, and the frequency-current relationship was examined in wild-type and med neurons. Figure 8A shows examples in the presence of 4-AP of typical discharge patterns for both groups of neurons evoked at three levels of current stimulation: just threshold stimulus intensity (40 pA), intermediate intensity (800 pA), and at a level that induced complete cessation of spike activity after the initial spike in all med neurons (1.8 nA). In all cases, the onset of spike block (reduction in number of spikes with increasing stimulus intensity; Table 3) and onset of complete spike inactivation for med neurons occurred at stimulus intensities below that for wild-type neurons (Fig. 8B, arrows). Figure 8B shows the frequency-current relationship for both the first ISI and steady-state discharge before the onset of spike block. Although the steady-state discharge frequencies were comparable at each current intensity for both groups before onset of spike block, the frequency of spike discharge just before the onset of spike block was lower for med neurons compared with wild-type neurons, thus
effectively narrowing the frequency-current relationship for the med neuron group. Therefore the data suggest that med neurons enter into spike inactivation at lower frequencies of discharge compared with wild-type neurons.

**DISCUSSION**

This study showed the importance of the properties of Nav1.6 channels in control of Mes V membrane excitability. We provided direct evidence for a role for persistent sodium current in amplification of membrane resonance, production of subthreshold oscillations, and subsequent burst discharge (Wu et al. 2001, 2005). Furthermore, the data support the previous proposal (Enomoto et al. 2006) that sodium channels with a resurgent sodium current mechanism participate in control of Mes V spike discharge. Importantly, the study showed that reduction of persistent sodium and resurgent currents in the med mouse dramatically affects the ability of these neurons to produce maintained rhythmic burst discharge.

**Contribution of Na\textsubscript{v}1.6 isoform to total sodium currents**

Based on voltage-clamp experiments using standard step pulse or ramp protocols, three components of sodium currents in med and wild-type neurons were observed, transient, persistent, and resurgent sodium currents, similar to that described in subthalamic neurons (Do and Bean 2003, 2004). Persistent and resurgent sodium current densities were reduced substantially (39 and 76%, respectively) compared with the peak fast transient sodium current (18% reduced) in med compared with wild-type neurons. The most parsimonious explanation is that Na\textsubscript{v}1.6 isoform is predominately, but not exclusively, responsible for the resurgent sodium current component and contributes substantially to \(I_{\text{NaP}}\). However, a caveat when using mutant animals is always the potential for up-regulation of other channels to maintain functional homeostasis. Thus the small change in fast transient current could reflect incomplete up-regulation of other Na\textsubscript{v} isoforms. However, the large change in the resurgent component in med neurons suggests minimal, if any, compensation of this component, similar to that shown in cerebellar Purkinje neurons (Raman et al. 1997), and large spinal dorsal root ganglion (DRG) sensory neurons (Cummins et al. 2005). Regardless of the degree of compensation, the lack of complete suppression of any of the sodium current components contributes to the altered excitability of mesencephalic trigeminal neurons in the med mutant mouse.

**FIG. 8.** In the presence of 1–3 4-aminopyridine (4-AP), med neurons exhibit spike block and complete inactivation at a lower current intensities compared with wild-type neurons. A: spike discharge in response to current pulses of increasing intensity for a wild-type and med neuron in the presence of 4-AP (50 \(\mu\)M). Note the strong reduction in spike amplitude and occurrence of spikes for mutant at 800 pA compared with wild-type. B: left: composite frequency-current plot for 1st interstimulus interval (ISI) for wild-type (\(n = 7\)) and med (\(n = 3\)) neurons. At \(-600\) pA, med neurons exhibited onset of spike block. Right: same relationship for steady-state discharge.

**TABLE 3. Discharge characteristics of Mes V neurons in 4-AP**

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<th>Maximum First ISI Frequency</th>
<th>Maximum Mean Inst. Frequency</th>
<th>Maximum Current</th>
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<tr>
<td>Wild-type</td>
<td>103.7 ± 18.8 (7)</td>
<td>79.6 ± 25.1 (7)</td>
<td>1457.1 ± 299.2 (7)</td>
</tr>
<tr>
<td>Mutant</td>
<td>48.7 ± 11.8 (3)*</td>
<td>44.2 ± 9.5 (3)+</td>
<td>433.3 ± 230.9 (3)*</td>
</tr>
</tbody>
</table>

Values are means ± SD with number of cells in parentheses for each group. Averaged group values were compared with Student’s \(t\)-test. *\(P < 0.01\), †\(P < 0.05\). Mes V, mesencephalic trigeminal. 4-AP, 1-3-4-aminopyridine; ISI, interstimulus interval.
current components indicates that they are not exclusive to NaV
1.6 channels (Do and Bean 2003). Conversely, expression of
NaV 1.6 does not guarantee the presence of Ires, as shown in a
number of different types of neurons (Garcia et al. 1998; Leao
et al. 2006; Pan and Beam 1999; Raman and Bean 1997).

Subthreshold membrane properties of med mice

This study showed that the kinetic properties of NaV 1.6 channels are important in subthreshold resonance and mem-
brane potential oscillations. Previously, we provided evidence
that sodium channels with persistent kinetics are important for
amplification of membrane resonance, production of sub-
threshold oscillations, and initiation and maintenance of con-
ditional burst discharge in Mes V neurons (Wu et al. 2001,
2005). Subsequently, using the action potential clamp method
(Do and Bean 2003), we showed that Ires and ISAP flow during the
peak of the spike afterhyperpolarization potential (AHP)
and ISI, respectively, during rhythmical burst discharge, and
sodium channels that exhibit these properties should contribute
to facilitation of repetitive spike discharge (Enomoto et al.
2006). This study showed that the properties of NaV 1.6 chan-
nels determined in voltage clamp by measuring the associated
transient, persistent, and resurgent components, i.e., their ten-
dency to remain open at subthreshold potentials and produce a
long-lasting conductance (gNaP), their relatively negative activ-
ation threshold, and rapid block and unblock, contribute to
shaping subthreshold behavior and maintaining spike dis-
charge normally. The presence of resonance in Mes V med
neurons is not surprising because it has been determined by the
interaction of a low threshold potassium current and the pas-
sive membrane time constant (RC) (reviewed in Hutcheon and
Yarom 2000; Wu et al. 2001). However, the magnitude of the
resonance, as indicated by the Q-value obtained from the
impedance-response curve, is amplified as a result of the
noninactivating property of NaV 1.6 channels and the rapid
block and unblock of those channels at subthreshold potentials,
which in voltage clamp is manifest predominately as ISAP
Reduction of ISAP by low doses of TTX or riluzole reduces the
Q-value and resonance, as shown in Mes V neurons experi-
mentally and computationally (Wu et al. 2001, 2005). In med
neurons, the Q-value was significantly reduced, and as a
consequence, subthreshold oscillations were attenuated, further
indicating that the noninactivating properties of NaV 1.6 chan-
nels serve to amplify resonance. Although we cannot rule out
the possibility that changes in other conductances contribute to
the observed reduction in the FRC in med mice, subthreshold
potassium conductances are unlikely because resting potential
and input resistance were similar for both groups.

Firing patterns and burst discharge in med mice

This is the first study to examine the discharge patterns of
primary sensory neurons in mice deficient in NaV 1.6. Recently,
it was shown that large sensory DRG neurons from med mice are
devoid of resurgent sodium currents (Cummins et al. 2005).
Because discharge patterns were not examined, the role of Ires
was not determined. In this study, the firing patterns (single spike,
phasic or tonic discharge) in response to a 1-s pulse or maintained
membrane depolarization were significantly compromised, and
the spike frequency was reduced modestly in med neurons com-
pared with wild-type neurons. In wild-type neurons in response to
a 1-s current pulse from −50 mV, tonic discharge was most often
observed, but in a minority of neurons, phasic discharge (rapid
adaptation), which results from activation of a low threshold,
voltag dependent 4-AP sensitive potassium current (Del Negro
and Chandler 1997; Wu et al. 2001) was obtained. However, in
med neurons, single spike or a short burst of a few spikes, as
opposed to tonic discharge, was commonly observed. Moreover,
maintained rhythmical burst discharge in response to a long,
depolarizing current pulse was completely absent in all med
neurons regardless of membrane potential. The change to less
excitable neurons could result, partly, from suppression of INaP,
which previously was shown to flow during repetitive discharge in
Mes V neurons (Enomoto et al. 2006), and/or a to the shift to the
right of V1/2max activation.

However, it is unlikely that reduction of INaP in med Mes V
neurons is solely responsible for the shift from tonic to phasic or
single spike discharge and to the absence of maintained rhythmical
burst discharge in those neurons. If the absence of a
maintained tonic depolarizing current was mainly responsi-
ble, an increase in extrinsic depolarizing current should
compensate for the reduction of INaP and reinstate tonic discharge
and/or rhythmical bursting (Khaliq et al. 2003). However, this
was not observed in Mes V med neurons, suggesting additional
factors are responsible for these changes. Although, changes in
other ionic currents as a result of the mutation were not measured in this study and could contribute to the decreased
excitability, reduction in Ires, as shown previously (Khaliq et al.
2003), is a likely significant factor.

Previously, it was shown that Ires is 1) responsible for a
short-term “boost” in inward current after spike repolarization
and, more importantly, 2) associated with rapid recovery from
sodium channel inactivation during modest hyperpolarization
during the AHP in cerebellar Purkinje neurons, thus maximiz-
ing the potential for maintained high-frequency spike discharge
(Khaliq et al. 2003; Raman et al. 1997). These results suggest
a similar role for Ires in Mes V neurons. In addition to
alterations in spike discharge pattern and reduced spike fre-
ceency in response to current stimuli, in the presence of 4-AP
to reduce low threshold K+ currents, and the associated mem-
brane shunt, med neurons entered into depolarizing spike block
(inability to show maintained spike discharge throughout the
stimulus pulse with increasing stimuli) at significantly lower
current intensities compared with wild-type neurons. This is
consistent with the hypothesis that med neurons are more
susceptible to entry into conventional sodium channel inacti-
vation and are slower to recover from this process at depolar-
ized potentials compared with wild-type mice. Med neurons
routinely produce complete sodium channel inactivation at
stimulus currents significantly lower than those observed in
wild-type neurons (Fig. 8). It is likely that maintained rhyth-
mical bursting at depolarized potentials was not possible be-
cause of insufficient availability of sodium channels after the
first spike. Thus the presence of a resurgent mechanism most
likely facilitates rapid recovery from inactivation and high-
frequency discharge in Mes V neurons, even in the presence of
modest AHP amplitudes during a spike train. Similar roles for
Ires were proposed previously in cerebellar Purkinje neurons
(Khaliq et al. 2003; Raman and Bean 1997, 2001).

Others suggested that the absence of resurgent current in
Purkinje neurons of NaV 1.6 null mice was caused by the faster
inactivation kinetics of the sodium channels by conventional inactivation that normally competes with (regulates) the endogenous open channel blocking particle (Grieco and Raman 2004). This was based on the observation that, in null mice, they were able to restore the resurgent current pharmacologically. They interpreted this to mean that in fact all sodium channel isoforms in Purkinje neurons are associated with an endogenous open channel blocking particle, but in null mice, the conventional inactivation mechanism is too fast to allow block to occur. Interestingly, although not studied in detail, we found that the time constants for inactivation for the fast transient current were similar in both wild-type and med mice, suggesting that, in contrast to Purkinje neurons, the large suppression of \( I_{\text{res}} \) in Mes V neurons from med mice results from the absence of an association of endogenous open channel blocking particle with non-Na\(_1\)6 sodium channel isoforms. Additional experiments are necessary to clarify this.

Mes V neurons are important in oral-motor pattern generation; traditionally, they function as primary sensory neurons relaying proprioceptive information from muscle spindles periodontal receptors but can also function as trigeminal interneurons because of their unique location within the brain stem. Although they are not responsible for the rhythm generation during rhythmic oral-motor activity, their ability to discharge at high frequencies and produce burst generation is likely important in aspects of oral-motor pattern generation such as rapid synchronization within the Mes V pool through electrical resonance and enhance subthreshold oscillations and initiate the burst mode. Once bursting ensues, the rapid block and unblock of these channels maintains the burst mode. Naturally occurring modulation of Mes V neuron Nav1.6 sodium channel conductances by various neuromessengers during oral-motor behavior will be a mechanism to alter the effects these neurons have on their targets and thus oral-motor pattern generation.  

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**References**


