Sodium Currents in mesencephalic trigeminal neurons from Na\textsubscript{1.6} null mice

Akifumi Enomoto \textsuperscript{1,2}, Juliette M. Han \textsuperscript{1}, Chie-Fang Hsiao \textsuperscript{1}, and Scott H. Chandler \textsuperscript{1}

\textsuperscript{1} Department of Physiological Science, University of California at Los Angeles, Los Angeles, CA, 90095, USA; \textsuperscript{2} 1\textsuperscript{st} Department of Oral Maxillofacial Surgery, Graduate School of Dentistry, Osaka University, Osaka, 565-0871, Japan.

Running Head: Sodium currents and burst generation

Correspondence to:
Scott H. Chandler PhD
Department of Physiological Science, UCLA, 2859 Slichter Hall, Los Angeles, CA 90095
Tel: 310-206-6636
E-mail: schandler@physci.ucla.edu

216 words in abstract
Number of figures: 8
Number of tables: 3
Number of pages: 22
Abstract

Previous studies using pharmacological methods suggest that subthreshold sodium currents are critical for rhythmical burst generation in mesencephalic trigeminal neurons (Mes V). In the present study we characterized transient (I_{NaT}), persistent (I_{NaP}) and resurgent (I_{res}) sodium currents in Na\(_{1.6}\)-null mouse (med mouse, Na\(_{1.6}^{-/-}\)) lacking expression of the sodium channel gene Scn8a. We found that peak transient, persistent and resurgent sodium currents from med (Na\(_{1.6}^{-/-}\)) mice were reduced by 18%, 39% and 76% relative to their wild-type (Na\(_{1.6}^{+/+}\)) 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 in spite of the similar passive membrane properties compared to 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 to wild-type neurons. Importantly, med neurons never exhibited maintained stimulus induced rhythmical burst discharge unlike those of wild-type littermates. The data show 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 non-inactivating sodium currents, termed persistent sodium currents (INaP), 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 amongst others). More recently, some neurons were found to exhibit an additional current called resurgent sodium current (Ires) (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 Ires is associated predominately with the presence of the NaV 1.6 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 rhythmical burst behavior upon membrane depolarization (Wu et al. 2001, 2005). The membrane resonance is dependent upon a low threshold, 4-AP sensitive potassium current and is amplified by INaP to produce subthreshold oscillations and bursting. A subsequent in vitro study showed that during a given burst, both INaP and Ires 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 NaV 1.6 (med mouse) (Burgess et al. 1995) that in Purkinje neurons reduces INaP and Ires substantially (Raman and Bean 1997). These mice show altered motor functions, such as ataxia and progressive paralysis prior to death, which occurs around 3 weeks of age (Meisler et al. 2001).
In this study, we found that, indeed, in med mice neurons, $I_{\text{NaP}}$ and $I_{\text{res}}$ 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.

Materials and methods

Heterozygous Scn8a$^{med}$ mice maintained in strain C3Heb/FeJ were obtained from Jackson Laboratories (Bar Harbor, ME). The med mutation produces complete loss of Na$_v$1.6 expression. For the initial experiments, genotyping was performed after the experiments. However, for most experiments in order to compare only homozygous null animals with wild-type animals and increase the efficiency of our experiments, genotyping was performed for mice before experiments, and we then selected only homozygous med (Na$_v$1.6$^{-/-}$) or wild-type (Na$_v$1.6$^{+/+}$) litter-mates.

After extracts from mouse tails were obtained the tails were incubated in 0.4 ml of lysis buffer (100mM NaCl, 10mM Tris pH 8.0, 25mM EDTA, 0.5% SDS, 0.1 mg/ml Proteinase K) in 50°C water-bath overnight. After the samples were centrifuged at 6,000 rpm for 10 minutes, the supernatant was transferred to a tube containing 400 ul 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, GGA GCA AGG TTC TAG GCA GCT TTA AGT GTG and GTC AAA GCC CCG GAC GTG CAC ACT CAT TCC (Kohrman et al. 1996) and for the mutant allele, TCC AAT GCT ATA CCA AAA GTC CC and GGA CGT GCA CAC TCA TTC CC (Integrated DNA technologies INC). The reaction consisted of 30 s at 94°C, 30 s at 57°, 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 mesencephalic trigeminal (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 oxygenated (95%O$_2$-5%CO$_2$) ice-cold cutting solution of the following composition (in mM): 126 NaCl, 3KCl, 1.25 NaH$_2$PO$_4$, 26 NaHCO$_3$, 10 glucose, 1 CaCl$_2$, 5 MgCl$_2$, and 4 lactic acid (Schurr et al. 1988). The brain stem was glued by its rostral end to the platform of a chamber and covered with ice-
cold cutting solution. Six slices (300 µm) were cut on a vibrating slicer (DSK microslicer, Ted Pella, Redding, CA) and placed at room temperature into an oxygenated incubation solution of the following composition (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 2 CaCl₂, 2 MgCl₂, and 4 lactic acid (Schurr et al. 1988). The slices were incubated at 37 ºC for 40-50 min and then maintained at room temperature (22-24 ºC).

**Whole-Cell Recording**

Patch electrodes were fabricated from borosilicate glass capillary tubing (1.5mm OD, 0.86mm ID) using a Model P-97 puller (Sutter Instrument Co., Novato, CA). The patch pipettes were coated near the tip with silicone agent (Sylgard, Dow Corning Co., Midland, MI) to reduce capacitance. Whole-cell current and voltage clamp recordings were performed with an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA) in concert with pCLAMP acquisition software (ver 9.2, Axon Instruments). Signals were grounded (Ag/AgCl wire) using a 3 M KCl agar bridge. Following the establishment of a gigaohm seal, the whole-cell configuration was obtained by brief suction. Cells with seals < 1 GΩ before breakthrough were discarded. Uncompensated series resistance was usually less than 10 MΩ, compensated 60 - 90 %, and monitored periodically throughout the experiment. The data were low-pass filtered at 10 kHz and sampled at 10-50 kHz depending on the nature of the experiment. The liquid junction potentials were measured directly by recording the voltage offset produced by sequentially immersing a patch electrode in the electrode solutions followed by ACSF (Wu et al., 2001; Zhang and Krnjevic 1993). This was -7mV and corrected off-line. Tip resistances were 3-5 MΩ 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 brainstem slices ~500 µm lateral to the midline. Mesencephalic 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 minutes 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-gluconate, 25 KCl, 9 NaCl, 10 Hepes, 0.2 EGTA, 1 MgCl₂,
3 K₂-ATP, and 1 Na-GTP, pH 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, 1 MgCl₂, 3 K₂-ATP, and 1 Na-GTP. The external solution contained (in mM): 131 NaCl, 10 HEPES, 3 KCl, 10 glucose, 1 CaCl₂, 2 MgCl₂, 10 tetraethylammonium (TEA)-Cl, 10 CsCl, 1-3 4-aminopyridine (4-AP), and 0.3 CdCl₂. Sodium currents were defined by subtraction of the currents remaining in 0.5 µM TTX. In some experiments, to measure the transient sodium current, the external Na⁺ concentration was reduced to 50mM and substituted with 91mM TEA to minimize series 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 sec 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 1-2 sec 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: $Z = \text{FFT}(V)/\text{FFT}(I)$. 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 ($F_{res}$) and the $Q$ value. The $F_{res}$ was defined as the frequency at the peak of the hump in the FRC. The $Q$ value was calculated by measuring the impedance at $F_{res}$ and dividing that by the magnitude of the impedance at the lowest frequency measured (usually 1Hz) (Koch 1984; Hutcheon et al. 1996). 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: $I(t) = a \sin(bt^3)$, $0 \leq t \leq T$. 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, $a = 5$, $b = 10^{-7}$, $t = 8$ sec, $T = 10$ sec. 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 were reported as mean ± SD, unless indicated otherwise. The group comparison of mean values was performed with 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) (Nav1.6$^{+/+}$) and med (Nav1.6$^{-/-}$) 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 mV to −30 mV, and maximal current was elicited by steps to around −25 mV. As shown in Figure 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 peak current was
reduced by 18% in cells from med mice (−21.1 ± 10.3 pA/pF, n = 8) compared to that of wild-type (−25.8 ± 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 mV. The midpoint for wild-type was −32.9 ± 0.2 mV while Med mice exhibited a 1.6 mV shift in the depolarizing direction (V_h = −31.3 ± 0.4 mV). Slope factors were similar in neurons from wild-type (k = 6.1 ± 0.2) and med mice (k = 6.2 ± 0.3). Although there was a small shift to the depolarizing direction, the voltage dependence of activation was not significantly different between two groups.

The voltage dependence for inactivation was measured by applying 100 ms pre-pulses 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 side bottom) and med mice (bottom right side) 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 ± 0.5, k = 9.5 ± 0.4, n = 5) and med mice (V_h = −61.2 ± 0.7, k = 9.4 ± 0.6, n = 5). Furthermore, the time constant for fast and slow inactivation at -30mV for both populations were similar (WT, tau_{fast} = 0.75 ± 0.24 ms, tau_{slow} = 3.60 ± 1.1 ms; med, tau_{fast} = 0.65 ± 0.11 ms, tau_{slow} = 3.58 ± 1.22 ms, n=8, p>0.05).

Persistent sodium current

To characterize persistent and resurgent sodium current components the following protocols were employed (Do and Bean 2003; Enomoto et al. 2006). Resurgent current was measured at -40mV following a brief step to +30mV. The amplitude of I_{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 side). 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_{NaP} 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/sec) (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 -40mV. The ramp protocol speed was sufficient to completely inactivate the transient Na\(^+\) current yet allow maintenance of the slowly or non-inactivating persistent component.

In Mes V neurons from wild-type mice, persistent sodium current activated around -75.0 ± 5.5 mV and showed a peak current at -46.4 ± 3.8 mV (n = 8), while 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 to 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, \(I/(1 + \exp[-(V - V_h)/k])\), where V is the test potential, \(V_h\) is the midpoint, and \(k\) is the slope factor in mV. The midpoint for wild-type was -61.2 ± 2.1 mV. Med mice demonstrated a 4.2 mV shift in the depolarizing direction, with \(V_h = -57.0 ± 2.9\) 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 -40mV for both groups. In addition to differences in total peak current, the current densities where reduced approximately 76% in med mice compared to 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_{res}, the kinetics of this current between the two groups varied more modestly. When neurons were repolarized from +30 to −40 mV, where I_{res} is maximal, the I_{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 discharge

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 approximately 2 mV, whereas cell capacitance was approximately 20% reduced in the med group. Input resistance, although greater in the mutant group, was not significantly different. In most wild-type neurons, constant-current depolarization of the membrane from resting potential produced subthreshold, voltage-dependent oscillations of 1-5 mV that in a subset of neurons (n= 12/30) could initiate rhythmical burst discharge if the membrane was sufficiently depolarized, as previously described for rat Mes V neurons (Wu et al. 2001). Figure 5A shows an example of this phenomenon. Bursting occurred when the membrane was depolarized to a critical value (Fig. 5A1). Figure 5A2 shows the voltage-dependent nature of the subthreshold oscillations. The fast Fourier transform (FFT) of the noise data is shown to the right of each record. As the membrane potential was depolarized, a clear peak in the FFT emerged. Regardless of whether bursting was initiated, wild-type mice always exhibited subthreshold oscillations, as previously demonstrated in rat Mes V neurons (Wu et al. 2001). However, in the med mice population, bursting could not be induced (n= 0/44). Figure 5B1 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. 5B2, table 2), and peaks in the FFT were very small. The power in the peak frequency of the FFT (see Materials and Methods) 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 to 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 Materials and 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 figure 6B shows the FRC (see Materials and Methods) constructed from such data. In 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 demonstrated in rat Mes V neurons (Wu et al. 2001). When measured at holding potentials between -66 mV 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 Na$_v$1.6 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 demonstrated experimentally and computationally that $I_{res}$ contributes to control of discharge frequency in cerebellar Purkinje neurons (Khaliq et al. 2003). Since $I_{res}$ is significantly reduced in Mes V neurons from med mice and previously we demonstrated that $I_{res}$ does flow during spike repolarization and the early part of the 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 second 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 prior to 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 6 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) (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), while 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 figure 7B. For those neurons that exhibited either phasic or tonic discharge the maximal 1st ISI in response to a suprathreshold 1 second 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 to 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 sec stimulus is plotted vs stimulus intensity for all neurons examined. As shown, wild-type neurons produced greater numbers of spikes prior to spike block compared to 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 to 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 to wild-type neurons.

Mesencephalic 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 following 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 (arrows in figure 8B). Figure 8B shows the frequency-current relationship for both the 1\textsuperscript{st} ISI and steady-state discharge prior to the onset of spike block. Although the steady-state discharge frequencies were comparable at each current intensity for both groups prior to onset of spike block, the frequency of spike discharge just prior to the onset of spike block was lower for med neurons compared to 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 to wild-type neurons.

**Discussion**

The present study demonstrates the importance of the properties of Na\textsubscript{v}1.6 channels in control of Mes V membrane excitability. We provide 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 demonstrates that reduction of persistent sodium and resurgent currents in the med mouse dramatically affects the ability of these neurons to produce maintained rhythmical 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\%, 76\%, respectively) compared to the peak fast transient sodium current (18\% reduced) in med compared to 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\textsubscript{Na,p}. 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 indicates that they are not exclusive to Na\textsubscript{V} 1.6 channels (Do and Bean 2003). Conversely, expression of Na\textsubscript{V} 1.6 does not guarantee the presence of I\textsubscript{res}, as demonstrated in a number of different types of neurons (Garcia et al. 1998; Leao et al. 2006; Pan et al. 1999; Raman and Bean 1997).

**Subthreshold membrane properties of med mice**

The present study demonstrates that the kinetic properties of Na\textsubscript{v}1.6 channels are important in subthreshold resonance and membrane potential oscillations. Previously, we provided evidence that sodium channels with persistent kinetics are important for amplification of membrane resonance, production of subthreshold oscillations, and initiation and maintenance of conditional burst discharge in Mes V neurons (Wu et al. 2001, 2005). Subsequently, using the action potential clamp method (Do and Bean 2003), we demonstrated that I\textsubscript{res} and I\textsubscript{NaP} flow during the peak of the spike AHP and interspike interval, respectively, during rhythmic burst discharge, and sodium channels that exhibit these properties should contribute to facilitation of repetitive spike discharge (Enomoto et al. 2006). The present study demonstrates that the properties of Na\textsubscript{v}1.6 channels determined in voltage clamp by measuring the associated transient, persistent and resurgent components, i.e.; their tendency to remain open at subthreshold potentials and produce a long-lasting conductance (g\textsubscript{NaP}), their relatively negative activation threshold, and rapid block and un-block, contribute to shaping subthreshold behavior and maintaining spike discharge, normally. The presence of resonance in Mes V med neurons is not surprising since it is determined by the interaction of a low threshold potassium current and the passive 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 non-inactivating property of Na\textsubscript{v}1.6 channels and the rapid block and unblock of those channels at subthreshold potentials, which in voltage clamp is manifest predominately as I\textsubscript{NaP}. Reduction of I\textsubscript{NaP} by low doses of TTX or riluzole reduces the Q-value and resonance, as demonstrated in Mes V neurons experimentally 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 non-inactivating properties of Na\textsubscript{v}1.6 channels 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 since 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 demonstrated that large sensory DRG neurons from med mice are devoid of resurgent sodium currents (Cummins et al. 2005). Since discharge patterns were not examined, the role of \( I_{\text{res}} \) was not determined. In the present study, the firing patterns (single spike, phasic or tonic discharge) in response to a 1 sec pulse or maintained membrane depolarization were significantly compromised and the spike frequency was reduced modestly in med neurons compared to wild-type neurons. In wild-type neurons in response to a one second current pulse from -50mV, tonic discharge was most often observed, but in a minority of neurons, phasic discharge (rapid adaptation), which results from activation of a low threshold, voltage 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 \( I_{\text{NaP}} \), 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 \( V_{1/2\text{max}} \) activation.

However, it is unlikely that reduction of \( I_{\text{NaP}} \) 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 responsible then an increase in extrinsic depolarizing current should compensate for the reduction of \( I_{\text{NaP}} \) and re-instate 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 \( I_{\text{res}} \), as demonstrated previously (Khaliq et al. 2003) is a likely significant factor.

Previously, it was shown that \( I_{\text{res}} \) 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 maximizing the potential for maintained high frequency spike discharge (Raman et al. 1997; Khaliq et al. 2003). The present results suggest a similar role for I_{res} in Mes V neurons. In addition to alterations in spike discharge pattern and reduced spike frequency in response to current stimuli, in the presence of 4-AP to reduce low threshold K^+ currents and the associated membrane 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 to wild-type neurons. This is consistent with the hypothesis that med neurons are more susceptible to entry into conventional sodium channel inactivation and are slower to recover from this process at depolarized potentials compared to 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 rhythmical bursting at depolarized potentials was not possible due to 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 I_{res} were proposed previously in cerebellar Purkinje neurons (Khaliq et al. 2003; Raman et al. 1997; Raman and Bean 2001).

Others suggested that the absence of resurgent current in Purkinje neurons of Na_v1.6 null mice was due to 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 upon 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_{res} in Mes V neurons from med mice results from the absence of an association of endogenous open channel blocking particle with non-Na_v1.6 sodium channel isoforms. Additional experiments are necessary to clarify this.

Mesencephalic 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 due to their unique location within the brainstem. Although they are not responsible for the rhythm generation during rhythmical 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 via electrical synapses (Baker and Llinas 1971) and powerful activation of their target neurons, such as trigeminal jaw closing motoneurons. The properties of Nav1.6 in Mes V neurons clearly shape the discharge patterns of these neurons. At subthreshold potentials these channels significantly impart a low threshold, non-inactivating component that serves to amplify membrane 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.

Acknowledgments

This work was supported by National Institute of Dental and Craniofacial Research DE 06193. We would like to thank Gowry Fernando for genotyping the mice. Address for reprint requests: S.H. Chandler, Dept. of Physiological Science, 2859 Slichter Hall, Los Angeles, CA 90095-1568 (E-mail: schandler@physci.ucla.edu).

References


Garcia KD, Sprunger LK, Meisler MH, and Beam KG. The sodium channel Scn8a is the major contributor to the postnatal developmental increase of sodium current density in spinal motoneurons. J Neurosci 18: 5234-5239, 1998.


Figure legends

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 sides) and inactivation (bottom, left and right sides) protocols in low extracellular sodium solution (15 mM). The duration of the conditioning pulse was 100 ms during the inactivation protocol (protocols are shown as insets). B: composite peak current-voltage relationship for the sodium currents obtained from med mice (open circles) and wild-type neurons (solid circles). C: composite conductance-voltage relationship of activation and inactivation obtained from med (open circle) and wild-type neurons (solid circle). The 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. The reversal potential ($E_{\text{rev}}$) of the 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.

Fig. 2

Persistent sodium current properties of med and wild-type neurons. A: persistent sodium current traces evoked by a command step to -40 mV (left) from +30 mV, and during a slow ramp (right) protocol in standard extracellular solution. The amplitude of the persistent sodium current measured at the end of the pulse to -40 mV is very similar to that measured during the ramp at -40 mV. B: typical current-voltage relationship for persistent sodium current from med (gray) and wild-type neurons (black). C: normalized conductance-voltage relationship for $I_{NaP}$ activation for med (open circles) and wild-type neurons (solid circles). The 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. The reversal potential ($E_{\text{rev}}$) of the peak transient currents was $+55$ mV. Boltzmann function fit to data for both groups is shown as solid (wild-type neurons) or dotted (med neurons) lines.

Fig. 3

Resurgent sodium current properties of med and wild-type neurons. A: the top trace shows the voltage protocol and the middle (wild-type neurons) and bottom (med neurons) traces show the current responses. The transient sodium current was evoked by a 10 msec step pulse from $-90$ mV to $+30$ mV. Resurgent sodium current was elicited when the membrane was repolarized to voltages between $-70$
and –10 mV after maximal fast inactivation occurred. B: relationship between peak resurgent sodium current versus repolarization potential is shown in the top graph. Time to peak of $I_{\text{res}}$ after repolarization (middle) and decay time constant (bottom) for resurgent currents are shown below, respectively. Open circles indicate med neurons and solid circles indicate wild-type neurons.

**Fig. 4**

Persistent and resurgent sodium current densities are reduced in med mice. Each current density was measured at -40 mV. $I_{\text{NaT}}$, $I_{\text{NaP}}$ and $I_{\text{NaR}}$ from med mice were reduced by 12%, 44 %, 75% relative to their wild-type littermates, respectively. *P<0.01 for persistent; **P<0.001 for resurgent sodium current.

**Fig. 5**

Membrane properties of Mes V neurons from wild-type and med mice. A1: maintained stimulation produces rhythmical burst discharge in wild-type neurons. Inset shows regions of box at faster times base and higher gain. A2: high gain records show membrane potentials and subthreshold oscillations at different holding potentials. Note the increase in amplitude of the oscillations as a function of membrane potential. Solid vertical bar indicates time of occurrence of truncated spikes. FFT analysis of membrane oscillations taken at corresponding membrane potentials from the same wild-type neuron is shown at right side. Note the different scales for the power. B1 and B2: same as A1 and A2 except taken from med mouse neuron. Note the low amplitude membrane potential oscillations and absence of development of distinct voltage-dependent peaks in the FFT histograms compared to wild-type neuron.

**Fig. 6**

Membrane resonance is reduced in med mice neurons. A: typical subthreshold membrane potential response to ZAP function in wild-type and med neurons. B: impedance-frequency response curves derived from data in A.

**Fig. 7**

Med mice neurons exhibit altered discharge patterns. A: typical discharge in response to 1 second current pulse for wild-type neuron and med neuron. B: bar chart of distribution of discharge modes (single spike, phasic and tonic discharge) for wild-type and med neurons. C: plot of number of spikes in
a one second current pulse as a function of stimulus intensity for wild-type and med neurons. Note that spike block occurred in all neurons above 600 pA.

**Fig. 8**

In the presence of 4-AP med neurons exhibit spike block and complete inactivation at a lower current intensities compared to 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 μM). Note the strong reduction in spike amplitude and occurrence of spikes for the mutant at 800pA compared to wild-type. B: (left side) composite frequency-current plot for 1st ISI for wild-type (n=7) and med(n=3) neurons. At approximately 600pA med neurons exhibited onset of spike block. Right side shows same relationship for steady-state discharge.
Fig. 2

A

B

C

Normalized conductance vs. Voltage (mV)

Wild-type

Mutant
Fig. 3

A

Wild-type

Mutant

B

Voltage (mV)

Current (pA)

Time to peak (ms)

Decay Tau (ms)

Wild-type

Mutant
Fig. 4

[Bar chart showing current density (pA/pF) for wild-type and Mutant in transient, persistent, and resurgent states.]
Fig. 6

A

Wild-type
-46 mV

Mutant
-46 mV

Input current

B

Impedance (MΩ)

Frequency (Hz)

Wild-type

Mutant
Fig. 7

A  Wild-type

Mutant

20 mV

400 pA

200 ms

B

# of cells

12

8

4

0

Wild-type  Mutant

T  S  P  T

C

# of spikes

80

60

40

20

0

-20

100  500  900

Current (pA)

Wild-type  Mutant
<table>
<thead>
<tr>
<th></th>
<th>Resting membrane potential (mV)</th>
<th>Input resistance (MΩ)</th>
<th>Cell capacitance (pF)</th>
</tr>
</thead>
<tbody>
<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 (50) *</td>
<td>131.1 ± 60.8 (57)</td>
<td>58.9 ± 17.7 (69) **</td>
</tr>
</tbody>
</table>

Values are means ± SD with number of cells in parentheses for each group. Averaged group values were compared with Student t-test; *P < 0.05, **P < 0.01.
Table 2. Resonant Properties of Mes V Neurons

<table>
<thead>
<tr>
<th></th>
<th>Amplitude</th>
<th>Q value</th>
<th>Freq (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>3.2 ± 1.5 (12)</td>
<td>2.4 ± 0.6 (7)</td>
<td>77.0 ± 13.1 (7)</td>
</tr>
<tr>
<td>Mutant</td>
<td>2.1 ± 0.8 (16) *</td>
<td>1.7 ± 0.5 (8) *</td>
<td>76.2 ± 22.0 (8)</td>
</tr>
</tbody>
</table>

Values are means ± SD with number of cells in parentheses for each group. Averaged group values were compared with Student t-test; *P < 0.05.
Table 3. Discharge characteristics of Mes V neurons in 4-AP

<table>
<thead>
<tr>
<th></th>
<th>Max 1st ISI freq</th>
<th>Max Mean inst. freq</th>
<th>Max current</th>
</tr>
</thead>
<tbody>
<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 t-test; *P < 0.05, **P < 0.01.