Regulation of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8 peripheral nerve Na\textsuperscript{+} channels by auxiliary β-subunits

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Voltage-gated Na\textsuperscript{+} (Na\textsubscript{v}) channels are responsible for the rising phase of action potentials in many excitable cells and consist of a pore-forming α-subunit and one or more auxiliary β-subunits. The present study investigated the regulation by the β-subunit of two Na\textsuperscript{+} channels (Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8) expressed in dorsal root ganglion (DRG) neurons. Single cell RT-PCR was used to show that Na\textsubscript{v}1.8, Na\textsubscript{v}1.6, and β\textsubscript{1}–β\textsubscript{4} subunits were widely expressed in individually harvested small-diameter DRG neurons. Coexpression experiments were used to assess the regulation of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8 by β-subunits. The β\textsubscript{1}-subunit induced a 2.3-fold increase in Na\textsuperscript{+} current density and hyperpolarizing shifts in the activation (−4 mV) and steady-state inactivation (−4.7 mV) of heterologously expressed Na\textsubscript{v}1.8 channels. The β\textsubscript{2}-subunit caused more pronounced shifts in activation (−6.7 mV) and inactivation (−9.3 mV) but did not alter the current density of cells expressing Na\textsubscript{v}1.8 channels. The β\textsubscript{2}-subunit did not alter Na\textsubscript{v}1.8 gating but significantly reduced the current density by 31%. This contrasted with Na\textsubscript{v}1.6, where the β-subunits were relatively weak regulators of channel function. One notable exception was the β\textsubscript{4}-subunit, which induced a hyperpolarizing shift in activation (−7.6 mV) but no change in the inactivation or current density of Na\textsubscript{v}1.6. The β-subunits differentially regulated the expression and gating of Na\textsubscript{v}1.8 and Na\textsubscript{v}1.6. To further investigate the underlying regulatory mechanism, β-subunit chimeras containing portions of the strongly regulating β\textsubscript{1}-subunit and the weakly regulating β\textsubscript{2}-subunit were generated. Chimeras retaining the COOH-terminal domain of the β\textsubscript{1}-subunit produced hyperpolarizing shifts in gating and increased the current density of Na\textsubscript{v}1.8, similar to that observed for wild-type β\textsubscript{1}-subunits. The intracellular COOH-terminal domain of the β\textsubscript{1}-subunit appeared to play an essential role in the regulation of Na\textsubscript{v}1.8 expression and gating.

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The β3-subunit is expressed at high levels in small-diameter DRG neurons and in the I/II and X layers of the spinal cord. The distribution of β3-subunits in DRG neurons and the CNS exhibits a complementary pattern with that of the β1-subunit (Morgan et al. 2000). Unlike β1- and β2-subunits, the β3-subunit has a clearer role in neuropathic pain in that β3-subunit mRNA and protein levels are upregulated in various neuropathic pain models (Shah et al. 2001; Takahashi et al. 2003). Furthermore, β3-subunit mutations are associated with early-onset lone atrial fibrillation, and β3-subunit-null mice exhibit cardiac ventricular electrophysiology abnormalities (Hakim et al. 2008; Olesen et al. 2010).

The β1- and β2-subunits share a similar expression pattern in the CNS, but the β3-subunit is more abundantly expressed in DRG neurons than the β2-subunit, with higher levels in large-diameter DRG neurons and lower levels in small- and intermediate-diameter neurons (Yu et al. 2003). The β3-subunit has been reported to induce negative shifts in the activation of several Na+ channel subtypes, including Na1.1, Na1.2, Na1.4, and Na1.6, indicating that the β3-subunit may modulate the electrical properties of neurons by allowing Na+ channels to activate at more negative voltages (Yu et al. 2003; Chen et al. 2008; Aman et al. 2009). Since a free peptide derived from its cystoplasmic tail replicates the action of the endogenous blocking protein, the β3-subunit may be indirectly involved in the generation of resurgent currents (Grieco et al. 2005). Furthermore, recent studies have shown that the β3-subunit plays a role in the pathophysiology of a cardiac disease, long QT syndrome type 3, and neurological Huntington’s disease (Oyama et al. 2006; Medeiros-Domingo et al. 2007).

Using single cell RT-PCR techniques, we show that a large percentage of these small-diameter neurons (40–60%) express β1-, β2-, and β3-subunits, whereas only ~10% express the β2-subunit. We investigated how these β-subunits modulate the expression and gating properties of two different Na+ channel subtypes: Na1.6 and Na1.8. We demonstrate that the β3-subunit induces a significant increase in the current density of Na1.8 but has no effect on the current density of Na1.6. In addition, the COOH-terminal domain of the β1-subunit is involved in the modulation of the Na1.8 channel based on the results of experiments with a β1 COOH-terminal deletion variant and β2/β2-subunit chimeras harboring various regions of the β1-subunit together with the entire β2-subunit.

MATERIALS AND METHODS

Preparation of DRG neurons. Seven-day-old rat pups were anesthetized with isoflurane before decapitation. The rats were handled in accordance with the principles and guidelines of the local animal care committee, from which we received approval. DRGs were harvested from all accessible levels. The ganglia were incubated for 30 min at 37°C in 2 ml of HBSS-HEPES containing 1.5 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) followed by 1 mg/ml trypsin (Sigma-Aldrich) for an additional 30 min. Trypsin was removed, and the ganglia were transferred to L-15 Leibovitz media supplemented with 1% FBS (GIBCO), 2 mM glutamine, 24 mM NaHCO3, 38 mM glucose, 2% penicillin-streptomycin (GIBCO), and 50 ng/ml nerve growth factor (Sigma-Aldrich). The ganglia were disrupted using fire-polished Pasteur pipettes, and dissociated neurons were placed in 35-mm dishes containing 2 ml of supplemented Leibovitz media.

Single cell RT-PCR. Intact neurons were harvested by drawing the cells into large-bore 20-μm-diameter pipettes containing 20 μl of RNase-free water and were rapidly frozen for further analysis. Random hexamer primers (65 ng, Invitrogen, Carlsbad, CA) were added to 10-μl aliquots of cell lysates, which were heated to 70°C for 3 min and rapidly cooled on ice. mRNA was reverse transcribed in a 25-μl reaction containing Moloney murine leukemia virus reverse transcriptase (200 units, Fisher Bioreagents), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.5 mM dNTPs, and RNase inhibitor (1 U/μl, Promega). The remaining 10-μl aliquots of cell lysate were treated in an identical fashion except that water was substituted for reverse transcriptase in the reaction mixture. Thereafter, the first-strand cDNA synthesized in the reactions with or without reverse transcriptase (1–3 μl) was amplified in two successive rounds of a standard PCR protocol (30 cycles each) using nested gene-specific primers for Na+ channel β1-β2-subunits. Primer sets were designed to span one or more exon-intron borders to eliminate the possibility of contamination by genomic DNA. The PCR amplification was based on Taq polymerase (Roche Biochemicals) and used the following protocol: 94°C/1 min, 55°C/0.5 min, and 72°C/1 min (30 cycles). Additional controls included blanks in which the PCR amplification was performed in the absence of added reaction mixture with and without reverse transcriptase and a full RT-PCR analysis of the reaction without reverse transcriptase, the PCR blank, and the bath solution immediately surrounding the harvested neurons. The PCR products were sequenced. The sizes of the cDNA amplicons were estimated by running the samples on 2% agarose gels, after which the DNA was purified (QIAEx II, Qiagen) and sequenced.

Gene transfections and cell cultures. Two human embryonic kidney (HEK)-293 cell lines stably expressing human Na1.6 and rat Na1.8 were used. Both HEK-293 cell lines were grown under standard tissue culture conditions (5% CO2, 37°C) in high-glucose DMEM supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (10 mg/ml; GIBCO-BRL). HEK-293 cell lines stably expressing β1-, β2- and β3-subunits were cloned in our laboratory as previously described (Vijayaragavan et al. 2004). The rat β1-subunit was a gift from Dr. Lori L. Isom (University of Michigan, Ann Arbor, MI). The Na+ channel β1-β2-subunits and CD8 (empty vector) were constructed in the pIRES vector (Invitrogen), respectively, (pIERS/CD8/β1, pIERS/CD8/β2, pIERS/CD8/β1/β2, and pIERS/CD8). HEK-293 cell lines stably expressing Na1.6,Na1.8 and Na1.8 were transiently transfected with the same amount of individual β1-β2-subunits or empty vector pIRES/CD8 DNA. Transient transfections were carried out using the calcium phosphate method as previously described (Zhao et al. 2007). Transfected cells were briefly preincubated with CD8 antibody-coated beads before currents were recorded (Dynabeads M450 CD8-a). HEK-293 cells expressing the pIRES/CD8/Bicistronic vector were decorated with CD8 beads, which were used to identify cells for recording currents (Zhao et al. 2007). The β3/β2-subunit chimeras (β2β311, β2β321, and β2β312) and COOH-terminal deletion variant (β111A) were generous gifts from Dr. Thomas Zimmer (Institute of Physiology II, Friedrich Schiller University, Jena, Germany). β111 contains the extracellular domain of the β2-subunit and the transmembrane and intracellular domains of the β1-subunit. β221 contains the extracellular and transmembrane domains of the β2-subunit and the intracellular domain of the β1-subunit. β112 contains the extracellular and transmembrane domains of the β1-subunit and the intracellular domain of the β2-subunit. β11A contains only the extracellular and transmembrane domains of the β1-subunit. The 41 amino acid residues that form the COOH-terminal tail were deleted (see Fig. 6A). β1β1α, β2β2α, β1β2α, and β2β1α were constructed in the pIRES vector for expression in mammalian cell lines (pIERS/CD8/β2β211, pIERS/CD8/β2β212, and pIERS/CD8/β111A) and were individually transfected into the Na1,8-expressing HEK-293 cell line. Transient transfections of the β3/β2-subunit chimeras and the COOH-terminal deletion variant were performed using the calcium phosphate method as previously described (Zhao et al. 2007). Transfected cells were identified for patch-clamp...
analysis by preincubation with CD8 antibody-coated beads, as mentioned above.

**Whole cell patch-clamp recordings.** Macromscopic Na\(^+\) currents from rat DRG neurons and HEK-293 stable cells were recorded using the whole cell configuration of the patch-clamp technique. For whole cell patch-clamp recordings of DRG neurons, the pipette solution was composed of (in mM) 100 CsF, 25 CsCl, 10 NaCl, 1 EGTA, and 10 HEPES (pH 7.4). The bath solution was composed of (in mM) 140 NaCl, 2 KCl, 1.5 CaCl\(_2\), 1 MgCl\(_2\), and 10 HEPES (pH 7.4). TTX was bath applied at a final concentration of 300 nM. For HEK-293 stable cells, the pipette solution was composed of (in mM) 5 NaCl, 135 CsF, 10 EGTA, and 10 Cs-HEPES. The pH was adjusted to 7.4 using 1 N CsOH. The bath solution was composed of (in mM) 150 NaCl, 2 KCl, 1.5 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, and 10 Na-HEPES. The pH was adjusted to 7.4 with 1 N NaOH.

The liquid junction potential was measured as described by Neher (1992) (+7 mV) and was consistent to the one calculated using pCLAMP (+7.1 mV, Molecular Devices, Union City, CA). To correct for this junction potential, the pipette voltage was held at −7 mV, and the pipette offset was zeroed before making a giga seal. After that, no additional correction was necessary, and the applied voltages are the reported voltages.

The recordings were taken exactly 10 min after the whole cell configuration was obtained to allow the current to stabilize and fully dialyze the cell with pipette solution. Na\(^+\) currents were recorded at room temperature (22–23°C). Command pulses were generated, and currents were recorded using pCLAMP software (version 8.0) and an Axopatch 200 amplifier (Molecular Devices). Patch electrodes were fashioned from borosilicate glass (Corning 8161) and coated with silicone elastomer (Sylgard, Dow-Corning, Midland, MI) to minimize stray capacitance. Current recordings were taken using low-resistance electrodes (<1 MΩ), and the series resistance was compensated at values of ≥80% to minimize patch-clamp errors. Whole cell currents were filtered at 5 kHz, digitized at 10 kHz, and stored on a microcomputer equipped with an analog-to-digital converter (Digidata 1300, Molecular Devices).

Average current-voltage curves were obtained by plotting the current density (in pA/pF) versus the voltage. For the construction of activation curves, Na\(^+\) conductance (\(G_{Na}\)) was calculated from the peak current (\(I_{peak}\)) using the following equation: \(G_{Na} = I_{peak}/(V - E_{Na})\), where \(V\) is the test potential and \(E_{Na}\) is the reversal potential. Normalized \(G_{Na}\) was plotted against the test potentials. For the construction of inactivation curves, the peak current was normalized relative to the maximal value and plotted against the conditioning pulse potential. Steady-state activation and inactivation curves were fit to a Boltzmann equation of the following form: \(G/G_{max} = 1/(1 + \exp(V_{1/2} - V)/k_v)\), where \(V\) is conductance, \(G_{max}\) is maximal conductance, \(I\) is peak current, \(I_{max}\) is maximal current, \(V_{1/2}\) is the voltage at which the channels are half-maximally activated or inactivated, and \(k_v\) is the slope factor. The window current results from the overlap of voltage-dependent activation and inactivation that determines a range of potentials (window) at which Na\(^+\) channels are nonactivated and available for activation. Using the \(V_{1/2}\) and \(k_v\) values of voltage-dependent activation and inactivation, the probability of Na\(^+\) channels being within the window was calculated using the following equation: \(1/(1 + \exp((V_{1/2} activation - V)/k_{v activation})) \times 1/(1 + \exp((V - V_{1/2} inactivation)/k_{v inactivation}))\).

**Analysis of electrophysiological data.** Data were analyzed using a combination of pCLAMP software (version 9.0, Molecular Devices), Microsoft Excel, and SigmaPlot for Windows (version 11.0, SPS, Chicago, IL). Data are expressed as means ± SE.

**RESULTS**

**Single cell analysis of Na\(^+\) channel and β-subunit expression in DRG sensory neurons.** Figure 1A shows whole cell Na\(^+\) currents of a typical small-diameter (<25 μm) DRG neuron before (control) and after bath application of 300 nM TTX. The slowly inactivating TTX-resistant Na\(^+\) current observed in this neuron is characteristic of Na\(_{1.8}\) channels, which are known to be preferentially expressed in small-diameter sensory neurons (Sangameswaran et al. 1996). Single cell RT-PCR was used to investigate the expression of Na\(_{1.8}\) channels and auxiliary β-subunits in this population. Figure 1B shows the analyses of 53 individually harvested neurons. A high percentage of these neurons (80–85%) expressed Na\(_{1.7}\), Na\(_{1.8}\), and Na\(_{1.9}\) channels, consistent with what has recently been reported for small-diameter sensory neurons (Ho and O’Leary 2010). Between 40% and 60% of these neurons expressed at least one \(\beta_1\), \(\beta_2\), or \(\beta_3\) subunit. Only a small percentage (17%) expressed the \(\beta_4\) subunit, suggesting that this subunit is not widely expressed in these neurons. These data also pro-
vided insights into the overlap of β-subunit expression. The β2-β4 combination (39% neurons) was most frequently observed followed by β1-β3 (28%), β1-β2 (22%), β2-β4 (13%), β3-β4 (9%), and β1-β4 (7%). Overall, the data indicate that β-subunits are differentially expressed in subpopulations of small-diameter neurons, where they may regulate the expression and gating properties of Na\textsubscript{v}1.7, Na\textsubscript{v}1.8, and Na\textsubscript{v}1.9 channels present in these neurons.

Regulation of expression levels of Na\textsubscript{v}1.8 by β\textsubscript{1–4} subunits of HEK-293 stable cells. Western blots showed no detectable endogenous expression of β1–β4-subunits in HEK-293 cells (Aman et al. 2009). HEK-293 cells are thus well suited for assessing the effect of auxiliary subunits or empty vector into HEK-293 cells stably expressing endogenous expression of Nav1.8 channels. For the β\textsubscript{1–4} coexpression group, Nav1.8 currents activated earlier at 55 mV and reached its maximum at −10 mV. The reversal potential for all groups was −80 mV, or 6.2 mV less than the calculated value (86.2 mV; Fig. 3A).

To test the impact of Na\textsuperscript{+} channel β-subunits on the expression of Na\textsubscript{v}1.8, we transiently transfected individual β\textsubscript{1–4}-subunits or empty vector into HEK-293 cells stably expressing Na\textsubscript{v}1.8. Figure 2 shows representative Na\textsubscript{v}1.8 currents recorded from the HEK-293 stable cell line transiently transfected with individual β\textsubscript{1–4}-subunits or empty vector and normalized to membrane capacitance. For all groups except for the β4-subunit coexpression group, Nav1.8 currents activated at approximately −45 mV, and the peak inward current occurred at approximately −5 to 5 mV. The β4-subunit coexpression group activated earlier at −55 mV and reached its maximum at −10 mV. The reversal potential for all groups was −80 mV, or 6.2 mV less than the calculated value (86.2 mV; Fig. 3A).

Fig. 2. Representative Na\textsubscript{v}1.8 current traces in human embryonic kidney (HEK)-293 stable cells transiently coexpressed with individual β\textsubscript{1–4} (A–D) and normalized to membrane capacitance. The inset in A shows the protocol. Currents were elicited by depolarizing steps between −100 and 90 mV in 5-mV increments for 50 ms. Cells were held at a holding potential of −140 mV.
Fig. 3. Whole cell Na,1.8 currents in HEK-293 stable cells transiently transfected with individual β1–β2-subunits or empty vector. A: current-voltage relationship of Na,1.8 transfected with individual β1 (n = 18), β2 (n = 21), β3 (n = 19), or β4 (n = 14)-subunits or empty vector (n = 15). Current densities were measured by normalizing current amplitudes to the membrane capacitance and were plotted versus voltage. The experimental protocol is the same as in Fig. 2A. B: histogram showing the average current densities of Na,1.8 coexpressed with different β-subunits or empty vector. When cells were depolarized to 0 mV, coexpression with the β1-subunit produced a 2.3-fold increase in Na,1.8 current density compared with the empty vector control (**P < 0.01). The β2- and β3-subunits caused no increase in Na,1.8 current density (P > 0.05), whereas the β4-subunit induced a 30% decrease in Na,1.8 current density (**P < 0.01). C: composite figure showing the steady-state activation and inactivation of Na,1.8 coexpressed with individual β1–β2-subunits or empty vector. For clarity, we only show the symbols of the empty vector control group and the group that exhibited a significant difference, and we removed the symbols of the other groups. As such, only the symbols for the β1, β2, and control groups are shown, and the symbols for the β3 and β4 groups have been removed. Activation curves were generated using the same protocol as in Fig. 2A. Coexpression of β1- or β2-subunits with Na,1.8 induced a hyperpolarizing shift of the activation curve from −15 to 10 mV for the β1 group (P < 0.01 or P < 0.05) and from −50 to 10 mV (P < 0.01) for the β2 group. Steady-state inactivation was determined using 20-ms test pulses to 15 mV after 500-ms prepulses to potentials ranging from −140 to 5 mV (see the inset under the inactivation curves for the protocol). Like voltage-dependent activation, coexpression of β1- or β2-subunits also caused a hyperpolarizing shift of the inactivation curves from −70 to −60 mV for the β1-subunit (P < 0.01 or P < 0.05) and from 0 to −30 mV for the β2-subunit (P < 0.01 or P < 0.05). Both the activation and inactivation curves were fitted to a single Boltzmann function (see MATERIALS AND METHODS). The midpoints (V1/2) and slope factors (k) of the activation and inactivation curves are shown in Table 1. D: effects of β1- and β2-subunits on the Na,1.8 window current. The activation and inactivation curves of the Na+ channels overlap at a (relatively narrow) distinct voltage range (window), predicting a steady Na+ conductance over this range. The probability of Na,1.8 channels being within this window was calculated using the equation given in MATERIALS AND METHODS. The β1-subunit decreased the window current, and the peak probability shifted to a more depolarized potential, whereas the β2-subunit slightly increased the window current of Na,1.8.

produced a significant −16.7-mV shift (−29.2 ± 1.7 mV, n = 14, P < 0.01).

To investigate the effects of β1–β2 subunits on steady-state Na,1.8 inactivation (Fig. 3C and Table 1), we applied a two-pulse voltage-clamp protocol composed of 500-ms prepulses to potentials between −140 mV and 5 mV followed by test pulses to 15 mV. Like steady-state activation, when individual β1–β2-subunits were coexpressed with Na,1.8, the β1- and β2-subunits caused −4.6-mV (P < 0.05) and −9.3-mV (P < 0.01) shifts in steady-state inactivation, respectively. Little modulation of voltage-dependent inactivation was observed when Na,1.8 was coexpressed with β2- or β3-subunits (P > 0.05).

The overlapping area of the steady-state activation and inactivation curves shown in Fig. 3C gives a range of potentials (window) at which some channels are in the open state but do not undergo fast inactivation. Na+ channels activated in this way cause “window currents” (Hodgkin et al. 1952). The leftward shifts in steady-state activation and inactivation caused by the β1- and β2-subunits should induce alterations in window currents. Figure 3D shows that the Na,1.8 channels were probably within this window, that is, were inactivated and were available for activation when coexpressed with β1- or β2-subunits. Compared with the empty vector control, the small but significant hyperpolarization caused by the β1-subunit shortened the window, indicating a lower probability of Na,1.8 being activated. The peak probability shifted to a more depolarized potential (β1: −36 mV and control: −42 mV), with a smaller peak probability (β2: 1.2% and control: 2.6%) and a smaller fraction of Na,1.8 channels (the sum of all probabilities) that would be activated near the resting membrane potential (β1: 0.43% and control: 0.73%). In contrast, the substantial leftward shift caused by the β2-subunit slightly enhanced the probability, with a peak probability of 2.8% at −41 mV and a larger fraction
Table 1. Effect of individual β-subunits on the activation and inactivation of Na\textsubscript{v}1.8 and Na\textsubscript{v}1.6 channels in HEK-293 cells

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<td>n</td>
<td>k\textsubscript{v}, mV</td>
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Values are means ± SE; n, number of cells. HEK-293 cells, human embryonic kidney-293 cells; V\textsubscript{1/2}, voltage at which Na\textsuperscript{+} channels are half-maximally activated or inactivated; k\textsubscript{v}, slope factor. *P < 0.05 and †P < 0.01 compared with the control (+empty vector) group.

(0.81%) of Na\textsubscript{v}1.8 channels that would be activated near the resting membrane potential.

Effect of β\textsubscript{1}–β\textsubscript{4}-subunits on the expression and gating properties of Na\textsubscript{v}1.6 in HEK-293 stable cells. To study the specificity of β-subunit regulation, we investigated the effect of β\textsubscript{1}–β\textsubscript{4} subunits on the expression of Na\textsubscript{v}1.6 in the HEK-293 stable cell line. We transiently transfected individual β\textsubscript{1}–β\textsubscript{4}-subunits or empty vector in HEK-293 cells stably expressing Na\textsubscript{v}1.6. Figure 4 shows representative current traces of Na\textsubscript{v}1.6 recorded from HEK-293 cells and normalized to membrane capacitance. Figure 5A shows the current-voltage relations of Na\textsubscript{v}1.6 currents. The activation threshold for these Na\textsubscript{v}1.6 currents was evoked with depolarizing voltage steps from −100 to 90 mV in 5-mV increments for 50 ms at a holding potential of −140 mV.
activation but did not appreciably alter the voltage dependence of activation. As such, only the 1-α-subunit produced an enhanced window current, as shown in Fig. 5C. Compared with the empty vector control, coexpression of the β3-subunit increased the opening probability of Na\textsubscript{1.6} within the window (β3: 0.26% and control: 0.15%). The peak probability of the β3-subunit group was shifted in a more hyperpolarizing direction (β3: −60 mV and control: −50 mV), with an increased fraction of Na\textsubscript{1.6} channels that would be activated near the resting membrane potential (β3: 0.08% and control: 0.045%).

When Na\textsuperscript{+} channels open transiently during recovery from inactivation, they generate a “resurgent current,” which has been reported to be carried mainly by Na\textsubscript{1.6} α-subunits. The cytoplasmic tail of the β3-subunit may be the endogenous open channel blocker responsible for the production of resurgent currents (Grieco et al. 2005; Raman and Bean 1997). However, we did not detect a resurgent current in Na\textsubscript{1.6}-expressing HEK-293 cells coexpressing the β3-subunit (Fig. 5D) or empty vector (data not shown). Similarly, no resurgent current was observed in Na\textsubscript{1.8}-expressing HEK-293 cells cotransfected with the β4-subunit (data not shown).

Fig. 5. Whole cell Na\textsubscript{1.6} currents in HEK-293 stable cells transiently transfected with individual β\textsubscript{1–3}-subunits or empty vector. A: current-voltage relations of Na\textsubscript{1.6} currents were obtained using the same protocol as in Fig. 4A. For clarity, we removed the overlapping symbols of the β\textsubscript{1–3}- and β4-subunit transfection groups and only kept their corresponding lines (β\textsubscript{1}: n = 9, β\textsubscript{2}: n = 8, β\textsubscript{3}: n = 11, and empty vector: n = 9). The β1-β4-subunits did not modulate the expression of Na\textsubscript{1.6} in HEK-293 cells. B: voltage dependence of activation and inactivation of Na\textsubscript{1.6} coexpressed with different β-subunits or empty vector. For clarity, we show only the symbols for the β3 and control groups and have removed the symbols for the other groups. The activation curves were generated using the same protocol as in Fig. 4A. Inactivation was measured using 500-ms prepulses to potentials between −140 and 5 mV. The fraction of available current was determined using test pulses to −20 mV at a holding potential of −140 mV (see the inset under the inactivation curve for the protocol). The smooth lines of activation and inactivation are fits to a Boltzmann function (see data analysis in MATERIALS AND METHODS). Coexpression of the β4-subunit caused a negative shift in the activation curve from −65 to −20 mV (P < 0.01 or P < 0.05). However, coexpression of β1-β3-subunits did not affect the voltage-dependent inactivation of Na\textsubscript{1.6} in HEK-293 cells. The values of V\textsubscript{1/2} and k\textsubscript{s} are shown in Table 1. C: effect of the β4-subunit on the Na\textsubscript{1.6} window current. The y-axis shows the probability of Na\textsubscript{1.6} channels being within the window, which was measured using the equation given in MATERIALS AND METHODS. Coexpression of the β4-subunit increased the probability of Na\textsubscript{1.6} opening within the window and shifted the peak probability in a more hyperpolarizing direction. D: representative resurgent Na\textsuperscript{+} current in HEK-293 cells coexpressing Na\textsubscript{1.6} and the β4-subunit. The voltage protocol used to elicit the currents is shown under the current trace. HEK-293 cells coexpressing Na\textsubscript{1.6} and the β4-subunit were depolarized from a holding potential of −120 mM by a prepulse to 30 mV for 40 ms to activate and inactivate Na\textsuperscript{+} channels. Each prepulse was followed by a single 100-ms test pulse to potentials ranging from −60 to 5 mV in 5-mV increments. Resurgent currents were not detected in all HEK-293 cells coexpressing Na\textsubscript{1.6} and the β4-subunit.
Differential regulation of the expression and gating properties of Na\textsubscript{1.8} in HEK-293 stable cells by \(\beta_1/\beta_2\)-subunit chimeras and a \(\beta_1\) COOH-terminal deletion variant. As indicated by our above results, only the \(\beta_1\)-subunit significantly increased the current density of Na\textsubscript{1.8} (2.3-fold) in HEK-293 cell stable cells. The \(\beta_2\)-subunit did not regulate the expression and gating properties of Na\textsubscript{1.8}. We took advantage of the nonmodulating \(\beta_2\)-subunit to investigate the molecular basis of \(\beta_1\)-subunit-mediated enhancement of Na\textsubscript{1.8} expression. Auxiliary \(\beta\)-subunits are transmembrane proteins composed of a NH\textsubscript{2}-terminal extracellular domain, a single transmembrane domain, and an intracellular COOH-terminal domain (Isom 2002a) (Fig. 6A, top). In the present study, we used \(\beta_1/\beta_2\)-subunit chimeras (\(\beta_{211}, \beta_{221}\) and \(\beta_{112}\)) and a \(\beta_1\) COOH-terminal deletion variant (\(\beta_{11\Delta}\)) to identify the molecular regions of the \(\beta_1\)-subunit that are involved in the modulation of Na\textsubscript{1.8} (Fig. 6A, bottom).

Coexpression of chimera \(\beta_{211}\), which was composed of the \(\beta_2\)-subunit extracellular domain and the \(\beta_1\)-subunit transmembrane and intracellular domains, increased the current density \((-155.8 \pm 12.2 \text{ pA/pF}, n = 14\) compared with control cells \((-73.9 \pm 5.7 \text{ pA/pF}, n = 15, P < 0.01\). To determine whether

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Fig. 6. A: schematic representation of the construction of \(\beta_1/\beta_2\)-subunit chimeras and the deletion mutant. Top, typical structure of wild-type \(\beta_1\) and \(\beta_2\)-subunits; bottom, the NH\textsubscript{2}-terminal (N), COOH-terminal (C), and transmembrane-spanning segments of the \(\beta_1\)-subunit were systematically replaced by corresponding segments of the \(\beta_2\)-subunit. \(\beta_{211}, \beta_{221}\), and \(\beta_{11\Delta}\) are \(\beta_1/\beta_2\)-subunit chimeras constructed by site-directed mutagenesis; \(\beta_{11\Delta}\) is a COOH-terminal deletion mutant of the \(\beta_1\)-subunit. B: current-voltage relationship showing the average current densities of Na\textsubscript{1.8} currents in HEK-293 stable cells transiently transfected with \(\beta_1\) (\(n = 14\), \(\beta_2\) (\(n = 13\), \(\beta_{211}\) (\(n = 14\), \(\beta_{221}\) (\(n = 9\), \(\beta_{11\Delta}\) (\(n = 14\), or \(\beta_{11\Delta}\) (\(n = 16\). The protocol is the same as in Fig. 2A. C: histogram showing the current densities of Na\textsubscript{1.8} channels transiently coexpressing wild-type, chimera, and mutant \(\beta\)-subunits. The current densities of the \(\beta_1\), \(\beta_{211}\), and \(\beta_{221}\) groups were significant larger than that of the empty vector control group (**\(P < 0.01\) but similar to each other. D: composite figure showing steady-state activation (right) and inactivation (left). The activation curves were generated using the same protocol as in Fig. 2A. Inactivation was measured using the same protocol as in Fig. 3C. The smooth lines of activation and inactivation are fits to a Boltzmann function (see data analysis in MATERIALS AND METHODS). For clarity, we show only the symbols for the \(\beta_1, \beta_{211}, \) and \(\beta_{221}\) groups, which exhibited significant differences compared with the empty vector group, and removed the symbols for other groups (\(\beta_2, \beta_{112}, \) and \(\beta_{11\Delta}\)). When \(\beta_1\), \(\beta_{211}\), or \(\beta_{221}\) were coexpressed with Na\textsubscript{1.8}, the steady-state activation curves exhibited a negative shift compared with the empty vector group (\(P < 0.01\) or \(P < 0.05\). When \(\beta_1\) or \(\beta_{221}\) were coexpressed with Na\textsubscript{1.8}, the steady-state inactivation curves exhibited a negative shift compared with the empty vector control group (\(P < 0.05\), whereas coexpression of \(\beta_2, \beta_{221}, \beta_{112}, \) and \(\beta_{11\Delta}\) with Na\textsubscript{1.8} did not have a significant effect on voltage-dependent inactivation (\(P > 0.05\)). The values of \(V_{1/2}\) and \(k_c\) are shown in Table 2.
the β₁-subunit intracellular domain was sufficient to modulate the current density of Naᵥ1.8, chimera β₂2₁, which was composed of the β₂-subunit extracellular and transmembrane domains and the β₁-subunit intracellular domain, was coexpressed with Naᵥ1.8. β₂2₁ increased the current density of Naᵥ1.8 (−152.7 ± 16.0 pA/pF, n = 9, P < 0.01). Current densities of the β₂2₁ and β₂2₂ groups were similar to the value observed with the β₁-subunit group (−164.8 ± 15.7 pA/pF, n = 14, P > 0.05). No increase in the current density of Naᵥ1.8 was observed in the β₁12 and β₁₁₄ groups, which contain the NH₂-terminus and transmembrane domain of the β₁-subunit but lack the COOH-terminus of the β₁-subunit (β₁₁₂: −81.5 ± 9 pA/pF, n = 14, and β₁₁₄: −87.2 ± 7 pA/pF, n = 16, P > 0.05; Fig. 6, B and C). This suggested that the increase in the peak current densities of Naᵥ1.8 observed in the wild-type β₁, β₂2₁, and β₂2₂ groups is due to the COOH-terminal region of the β₁-subunit.

To determine whether the increase in the peak current densities of Naᵥ1.8 caused by COOH-terminal of the β₁-subunit was accompanied by changes in the biophysical properties of Naᵥ1.8, the voltage dependence of activation and inactivation of wild-type channels, chimeras, and the deletion variant were studied (Fig. 6D). Compared with the empty vector control group, the β₁, β₂2₁, and β₂2₂ groups produced significant negative shifts in the V₁/₂ values of the activation curves by 5.2, 4.8, and 6.6 mV (P < 0.01 or P < 0.05), respectively, and in the V₁/₂ values of the inactivation curves by 4.5, 4.9, and 5.8 mV (P < 0.05), respectively. No significant differences in the voltage dependence of activation and inactivation were observed among the β₁, β₂2₁, and β₂2₂ groups.

DISCUSSION

The main goal of the present study was to investigate the regulation of Naᵥ1.6 and Naᵥ1.8 channels by auxiliary β-subunits. These Na⁺ channels are widely expressed in primary sensory neurons, where they contribute to the rapid rising phase of action potentials (Chahine et al. 2005). We used a combination of single cell RT-PCR of acutely dissociated DRG neurons and heterologous expression experiments to identify the β-subunits expressed in small-diameter sensory neurons and to investigate their regulation of functional Na⁺ channels, which are known to be expressed in these neurons. Our results indicated that small-diameter DRG neurons widely express Naᵥ1.6 and Naᵥ1.8 channels and auxiliary β₁β₂-subunits. These findings are in agreement with previous work using RT-PCR, in situ hybridization, and double labeling coupled with immunohistochemistry (Morgan et al. 2000; Takahashi et al. 2003; Yu et al. 2003).

Regulation of Naᵥ1.8 by auxiliary β-subunits. The single cell RT-PCR analysis of acutely dissociated DRG neurons indicated that Naᵥ1.8 channels are widely expressed in small-diameter neurons (Fig. 1B), which is consistent with recent work showing that the TTX-resistant Naᵥ1.8 channel is highly expressed in unmyelinated C-fibers (Ho and O’Leary 2010). These neurons also express β₁β₁, β₁β₂, and β₁β₂-subunits, raising the possibility that these auxiliary subunits may associate with and regulate endogenous Naᵥ1.8 channels expressed in these neurons. To further investigate this potential regulation, Naᵥ1.8 and β-subunits were coexpressed in HEK-293 cells, and the biophysical properties of the channel complexes were assessed using an electrophysiology approach. In the absence of auxiliary subunits, heterologously expressed Naᵥ1.8 channels produced robust Na⁺ currents that activated at relatively depolarized voltages (−50 mV) and displayed properties similar to the dominant TTX-resistant Na⁺ current of DRG neurons (Zhao et al. 2007). Coexpression of the β₁-subunit increased the current density twofold and produced hyperpolarizing shifts in activation (−4 mV) and steady-state inactivation (−4.6 mV). These shifts produced a twofold reduction in the window current, resulting in a decrease in the fraction of Naᵥ1.8 channels predicted to be open at voltages near the resting membrane potential (approximately −65 mV) of sensory neurons (Zhao et al. 2007). Our results indicated that Naᵥ1.8 and the β₁-subunit are coexpressed in the same population of DRG sensory neurons and that the association with the β₁-subunit increases the cell surface expression and alters the gating of the channels. Coexpression of the β₂-subunit did not alter Naᵥ1.8 gating but produced a 31% decrease in the peak current density (Fig. 3B). Whereas β₂ subunits were widely expressed in small-diameter DRG neurons (47%), this auxiliary subunit did not significantly alter the expression or gating of heterologously expressed Naᵥ1.8 channels. The β₂-subunit produced dramatic shifts in activation (−16.7 mV) and inactivation (−9.3 mV), resulting in an increase in the window current. These changes predicted that Naᵥ1.8 + β₂ channels would activate at more hyperpolarized membrane potentials and would increase the likelihood of a persistent Na⁺ current at more hyperpolarized voltages. β₂-Subunit regulation may increase the excitability of DRG neurons expressing this combination of subunits. However, unlike the β₁-subunit, the β₂-subunit was expressed in a relatively small percentage (17%) of small-diameter sensory neurons. This suggested that, although β₂-subunits are potent regulators of Naᵥ1.8 channels, they are expressed in a small subpopulation of unmyelinated C-fibers.

Regulation of Naᵥ1.6 by auxiliary β-subunits. Naᵥ1.6 channels are mainly expressed in large-diameter (>30 μm) myelinated sensory neurons (Ho and O’Leary 2010), where they are predominantly located at the nodes of Ranvier (Krzemien et al. 2000; Caldwell et al. 2000). Heterologously expressed Naᵥ1.6 channels generate a rapidly inactivating TTX-sensitive current that activates at a relatively hyperpolarized (−60 mV) voltage (Fig. 5A). The β₁-subunit and Naᵥ1.6 have reciprocal functions, such that β₁-subunit-mediated neurite outgrowth requires Na⁺ current carried by Naᵥ1.6, and the β₁-subunit is required for normal expression/high-frequency action potential firing of Naᵥ1.6 at the axon initial segment (Brackenbury et al. 2010). Our results showed that coexpression of β₁-subunits (β₁β₁β₂) does not alter the peak current density or voltage-dependent gating of heterologously expressed Naᵥ1.6 channels (Fig. 5). The sole exception was the β₂-subunit, which produced a hyperpolarizing shift (−7.6 mV) in Naᵥ1.6 activation. This may be significant because it resulted in a twofold increase in the window current and shifted Naᵥ1.6 activation into a range of voltages considered to be near the resting membrane potential of sensory neurons. These changes may increase the excitability of Naᵥ1.6 channels, leading to a reduction in the threshold for initiating action potentials in large-diameter, low-threshold sensory neurons.

The COOH-terminal domain of the β₁-subunit is critical for Naᵥ1.8 regulation. Previous studies have used β-subunit chimeras to identify the structural domains of the auxiliary sub-
units required for regulating Na\(^+\) channel function. Early studies of the neuronal Na\(_{1.2}\) channel indicated that the NH\(_2\)-terminus of the \(\beta_1\)-subunit is sufficient to fully recapitulate the accelerated inactivation, increased expression, and shifts in voltage-dependent activation and inactivation produced by the full-length \(\beta_1\)-subunit (McCormick et al. 1998). A similar role for the NH\(_2\)-terminal domain of the \(\beta_1\)-subunit has been previously postulated for the skeletal muscle Na\(_{1.4}\) channel (Chen and Cannon 1995). These findings were further supported by studies showing that the regions important for \(\alpha-\beta_1\) interactions are located within the extracellular loops of Na\(_{1.2}\) and Na\(_{1.4}\) channels (Makita et al. 1996; Qu et al. 1999). Subsequent work has implicated the intracellular COOH-terminal domain of the \(\beta_1\)-subunit as another important determinant of \(\alpha-\beta_1\) interactions and of Na\(_{1.2}\) regulation (Meadows et al. 2001). Current evidence suggests that both NH\(_2\)- and COOH-terminals of the \(\beta_1\)-subunit contribute to \(\alpha-\beta_1\) interactions and the functional regulation of neuronal and skeletal muscle Na\(^+\) channels. These findings contrast sharply with the results of studies on the cardiac Nav1.5 channel, where the membrane-spanning domain coupled with secondary interactions with either the NH\(_2\)- or COOH-terminal of the \(\beta_1\)-subunit were reported to be required for Na\(_{1.5}\) regulation (Zimmer and Benndorf 2002). These results point to substantial differences in the mechanisms of \(\beta_1\)-subunit regulation of neuronal, skeletal muscle, and cardiac Na\(^+\) channels.

We used chimeras of the strongly regulating \(\beta_1\)-subunit and weakly regulating \(\beta_2\)-subunit to identify the structural domains required for Na\(_{1.8}\) regulation. The intracellular COOH-terminal domain of the \(\beta_1\)-subunit appeared to be required for the increase in Na\(^+\) current density and the hyperpolarizing shifts in the activation and inactivation of Na\(_{1.8}\) channels. The observed changes in expression and gating were not altered by replacing the extracellular NH\(_2\)-terminus or membrane-spanning domains of the \(\beta_1\)-subunit with those of the \(\beta_2\)-subunit or by deleting the COOH-terminus of the \(\beta_1\)-subunit. These findings differ substantially from previous studies of neuronal and skeletal muscle Na\(^+\) channels, where the extracellular domain of the \(\beta_1\)-subunit appeared to play a more prominent role in Na\(^+\) channel regulation. Comunnoprecipitation studies have identified sites in the COOH-terminus of the \(\beta_1\)-subunit and neuronal Na\(_{1.1}\) channels that directly contribute to \(\alpha-\beta_1\) interactions (Spampanato et al. 2004). A similar interaction between the intracellular COOH-terminus of the \(\beta_1\)-subunit and Na\(_{1.8}\) may contribute to the observed increase in the expression and functional regulation of these channels.

The \(\beta_1\)- and \(\beta_2\)-subunits are both cell adhesion molecules that interact in a trans-homophilic fashion, resulting in ankyrin recruitment to the plasma membrane at points of cell-cell contact. Only the \(\beta_1\)-subunit can heterophilically interact with contactin, leading to increased surface expression of Na\(_{1.2}\) in CHL cells. \(\beta_1/\beta_2\)-Subunit chimeric studies in which the various regions of the \(\beta_1\) Ig loop were exchanged showed that the \(\beta_1\) Ig loop is not enough to induce full \(\beta_1\)-subunit-mediated enhancement of the Na\(_{1.2}\) cell surface (McEwen et al. 2004; Malhotra et al. 2000). Further studies showed that ankyrin recruitment by the \(\beta_1\)-subunit depends on the phosphorylation of \(\beta_1\)Y181, an intracellular tyrosine residue. A mutant of this residue (\(\beta_1\)Y181E) inhibits \(\beta_1\)-subunit-mediated ankyrin recruitment in response to homophilic adhesion and enhancement of Na\(_{1.2}\) surface expression (Malhotra et al. 2002; McEwen et al. 2004). While the \(\beta_1\)-subunit extracellular domain is homologous to the \(\beta_1\)-subunit, the \(\beta_2\)-subunit does not mediate the trans-homophilic cell adhesion that results in ankyrin recruitment (McEwen et al. 2009). Taken together, these findings provide support for the hypothesis that the COOH-terminus of the \(\beta_1\)-subunit plays an essential role in the modulation of Na\(^+\) channel function (McEwen et al. 2009).

Previous studies of \(\beta\)-subunit regulation. Previous studies have investigated the regulation of Na\(_{1.8}\) channels by auxiliary \(\beta\)-subunits. Early work examining Na\(_{1.8}\) channels expressed in Xenopus oocytes failed to detect changes in current kinetics when the channels were coexpressed with the \(\beta_1\)-subunit (Akopian et al. 1996; Sangameswaran et al. 1996). However, subsequent studies found that coexpression of the \(\beta_1\)-subunit in oocytes accelerates inactivation kinetics, increases the current density, and produces hyperpolarizing shifts in the activation and steady-state inactivation of Na\(_{1.8}\) channels (Vijayaragavan et al. 2004). A similar \(\beta_1\)-subunit-induced shift in activation was reported for the heterologously expressed human Na\(_{1.8}\) channel (Rabert et al. 1998). These observations are consistent with both our results and recent work showing that the \(\beta_1\)-subunit increases the current density and produces hyperpolarizing shifts in the gating of Na\(_{1.8}\) channels expressed in mammalian cells (Zhao et al. 2007).

There are conflicting results concerning the regulation of Na\(_{1.8}\) channels by the \(\beta_2\)-subunit. Coexpression of the \(\beta_2\)-subunit in oocytes results in an increase in Na\(_{1.8}\) current and a hyperpolarizing shift in activation (Shah et al. 2000). This contrasts with previous work showing that coexpression of the \(\beta_2\)-subunit in oocytes produces a depolarizing shift in Na\(_{1.8}\) inactivation but no change in current density (Vijayaragavan et al. 2004). \(\beta_2\)-Subunits have been reported to directly bind to 

### Table 2. Effect of \(\beta_1/\beta_2\)-subunit chimeras and \(\beta_1\) COOH-terminal deletion mutant on the activation and inactivation of Na\(_{1.8}\) channels in HEK-293 cells

<table>
<thead>
<tr>
<th></th>
<th>Activation</th>
<th>Steady-State Inactivation</th>
</tr>
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<tbody>
<tr>
<td>(V_{1/2}), mV</td>
<td>(k_+), mV</td>
<td>(n)</td>
</tr>
<tr>
<td>Na(_{1.8}) + (\beta_1)</td>
<td>-17.7 ± 1.8*</td>
<td>14</td>
</tr>
<tr>
<td>Na(_{1.8}) + (\beta_2)</td>
<td>-14.4 ± 1.2</td>
<td>13</td>
</tr>
<tr>
<td>Na(_{1.8}) + (\beta_2)</td>
<td>-17.8 ± 1.5*</td>
<td>14</td>
</tr>
<tr>
<td>Na(_{1.8}) + (\beta_2)</td>
<td>-19.1 ± 1.1†</td>
<td>9</td>
</tr>
<tr>
<td>Na(_{1.8}) + (\beta_2)</td>
<td>-14.5 ± 2.0</td>
<td>14</td>
</tr>
<tr>
<td>Na(_{1.8}) + (\beta_2)</td>
<td>-15.7 ± 2.1</td>
<td>16</td>
</tr>
<tr>
<td>Na(_{1.8}) + (\beta_2)</td>
<td>-15.7 ± 2.1</td>
<td>16</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\), number of cells. *\(P < 0.05\) and †\(P < 0.01\) compared with the Na\(_{1.8}\) + empty vector group (values are shown in Table 1).
Na\textsubscript{v}1.8 channels via the COOH-terminus of the \textbeta\textsubscript{3}-subunit and to help translocate Na\textsubscript{v}1.8 from the endoplasmic reticulum to the plasma membrane (Zhang et al. 2008). However, in the present study, coexpression of the \textbeta\textsubscript{3}-subunit in mammalian cells produced a significant 31\% decrease in Na\textsubscript{v}1.8 current density (Fig. 3B). This result is in agreement with a previous study showing that \textbeta\textsubscript{3}-subunits do not improve the functional expression of Na\textsubscript{v}1.8 in COS-7 cells (Swanwick et al. 2010). Collectively, these findings suggest that coexpression of the \textbeta\textsubscript{3}-subunit either has no effect or reduces Na\textsubscript{v}1.8 current without altering voltage dependence or gating kinetics. The role of the \textbeta\textsubscript{3}-subunit in neuropathic pain is closely associated with Na\textsubscript{v}1.3, both of which are upregulated after axotomy in a coordinated fashion. They have also been shown to be highly coexpressed in DRG neurons using the double-labeling method (Takahashi et al. 2003). The \textbeta\textsubscript{3}-subunit depolarizes the voltage-dependent activation and inactivation of Na\textsubscript{v}1.3 when expressed in HEK-293 cells and induces biphasic components of the inactivation curves, increasing the proportion of channels with slower inactivation kinetics (Cusdin et al. 2010).

The \textbeta\textsubscript{2} and \textbeta\textsubscript{3}-subunits share 35\% sequence similarity, and both subunits covalently associate with Na\textsuperscript{+} channels via disulfide bonds (Yu et al. 2003). In the present study, the \textbeta\textsubscript{2}-subunit did not alter the expression, kinetics, or voltage dependence of Na\textsubscript{v}1.8 channels (Table 1). This is in good agreement with previous work showing that the \textbeta\textsubscript{2}-subunit does not regulate Na\textsubscript{v}1.8 channels expressed in Xenopus oocytes (Vijayaragavan et al. 2004). In contrast, the \textbeta\textsubscript{3}-subunit produced hyperpolarizing shifts in the activation of Na\textsubscript{v}1.8 and Na\textsubscript{v}1.6 channels. Similar changes in activation have been reported for Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.4, and Na\textsubscript{v}1.6 channels coexpressed with \textbeta\textsubscript{2}-subunits (Chen et al. 2008; Yu et al. 2003; Aman et al. 2009). The hyperpolarizing shift in activation produced by the \textbeta\textsubscript{3}-subunit expanded the predicted Na\textsubscript{v}1.8 window current (Fig. 3D) and increased the likelihood of Na\textsubscript{v}1.8 activation and persistent TTX-resistant Na\textsuperscript{+} currents at hyperpolarized voltages. While the COOH-terminal domain of the \textbeta\textsubscript{3}-subunit has been proposed to act as the endogenous open channel blocker of Na\textsuperscript{+} channels (Grieco et al. 2005), we could not detect a reduction in current density or resurgent currents when Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8 channels were coexpressed with the \textbeta\textsubscript{3}-subunit.

**Conclusions.** The present study examined the functional regulation of neuronal Na\textsubscript{v}1.6 and sensory neuron-specific Na\textsubscript{v}1.8 channels by auxiliary \textbeta-subunits. Single cell RT-PCR revealed that the Na\textsubscript{v}1.8 channel and several \textbeta-subunits (\textbeta\textsubscript{1}, \textbeta\textsubscript{2}, and \textbeta\textsubscript{3}) were coexpressed in the same population of small-diameter neurons. The high level expression of TTX-resistant Na\textsuperscript{+} currents and the preferential expression of Na\textsubscript{v}1.7, Na\textsubscript{v}1.8, and Na\textsubscript{v}1.9 transcripts in these neurons were consistent with what has been reported for unmyelinated C-fibers. Association with the \textbeta\textsubscript{3}-subunit increased Na\textsuperscript{+} current density and produced shifts in gating, leading to Na\textsubscript{v}1.8 activation at voltages near the resting membrane potential of sensory neurons. The predicted increase in TTX-resistant Na\textsuperscript{+} currents could have important implications for the electrical excitability of sensory neurons. Previous work has shown that \textbeta\textsubscript{1}-subunits are predominately expressed in medium- and large-diameter DRG neurons, a pattern that is not altered in animal models of nerve injury (Oh et al. 1995; Takahashi et al. 2003). While Na\textsubscript{v}1.8 channels and \textbeta\textsubscript{1}-subunits are differentially expressed in small- and large-diameter sensory neurons, these subunits may overlap in subpopulations of these neurons (Ho and O‘Leary 2010; Oh et al. 1995; Takahashi et al. 2003). Our single cell analysis showed that 44\% of the neurons expressing Na\textsubscript{v}1.8 also express transcripts coding for the \textbeta\textsubscript{1}-subunit, which provides support for this possibility. Further studies are required to determine whether these \textbeta\textsubscript{1}-subunits are associated with Na\textsubscript{v}1.8 channels and whether they regulate the expression and gating of Na\textsubscript{v}1.8 channels in these neurons. (Table 2).


