Differential expression of sodium channel β subunits in dorsal root ganglion sensory neurons*

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*Running Title: Differential expression of β subunits in DRG neurons

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Background: Auxiliary β subunits regulate the voltage-gated sodium channels of DRG neurons. 

Results: β subunits are differentially expressed in subpopulations of DRG neurons and regulate Na,1.7 channels in an isoform-specific manner.

Conclusions: Differential β subunit expression and isoform-specific regulation has important implications for the sodium currents of DRG neurons.

Significance: β subunits are important determinants of sodium channel function and sensory neuron excitability.

SUMMARY

The small- (<25 μm) and large-diameter (>30 μm) sensory neurons of the dorsal root ganglion (DRG) express distinct combinations of tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium (Na⁺) channels that underlie the unique electrical properties of these neurons. In vivo these Na⁺ channels are formed as complexes of pore-forming α and auxiliary β subunits. The goal of this study was to investigate the expression of β subunits in DRG sensory neurons. Quantitative single-cell RT-PCR revealed that β subunit mRNAs were significantly correlated in small (β₂, β₃) and large (β₁, β₂) DRG neurons indicating that these subunits are co-expressed in the same populations. Co-immunoprecipitation and immunocytochemistry indicate that Na,1.7 forms stable complexes with the β₁, β₂ and β₃ subunits in vivo and that Na,1.7 and β₃ co-localize within the plasma membranes of small DRG neurons. Heterologous expression studies show that β₃ induces a hyperpolarizing shift in Na,1.7 activation while β₁ produces a depolarizing shift in inactivation and faster recovery. The data indicate that β₃ and β₁ subunits are preferentially expressed in small and large DRG neurons respectively and that these auxiliary subunits differentially regulate the gating properties of Na,1.7 channels.

The sensory neurons of the dorsal root ganglia (DRG) give rise to nerve fibers that convey information about thermal, mechanical, and chemical stimulation from peripheral tissues to the central nervous system. These neurons express a unique combination of tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium (Na⁺) currents that produce the rapid rising phase of the action potentials. Much of what is currently known about Na⁺ channel expression in sensory neurons is derived from electrophysiological studies of cultured DRG neurons (1-3). The small-diameter neurons (<25 μm) isolated from the DRG represent the cell bodies of unmyelinated nociceptors and preferentially express TTX-R Na⁺ current while the large-diameter (>30 μm) neurons, typically associated with low-threshold mechanoreceptors,
predominately express TTX-S Na$^+$ current. DRG sensory neurons express at least 6 distinct Na$^+$ channel isoforms that display properties similar to the endogenous TTX-S (Na$^+_{1,1}$, Na$^+_{1,2}$, Na$^+_{1,6}$, Na$^+_{1,7}$) and TTX-R (Na$^+_{1,8}$, Na$^+_{1,9}$) Na$^+$ currents observed in these neurons (4-7).

In vivo, voltage-gated sodium channels form complexes with auxiliary β subunits that regulate the trafficking, gating properties and kinetics of the endogenous Na$^+$ channels (8-12). β subunits are relatively small proteins (33-36 kDa) composed of a single membrane-spanning α-helix, a short intracellular C-terminus, and an large extracellular N-terminus incorporating an immunoglobulin-like fold similar to that found in adhesion molecules (8;13). Immunocytochemistry and in situ hybridization indicate that all four isoforms of β subunits (β1-β4) are expressed in sensory neurons (12;14;15).

This study employed a combination of single-cell RT-PCR, immunocytochemistry, immunoprecipitation and electrophysiology to further investigate β subunit expression in DRG sensory neurons. The data indicate that small and large DRG neurons express different complements of β subunits. The functional consequences of β subunit expression was evaluated by examining their regulation of Na$^+_{1,7}$, a TTX-S Na$^+$ channel widely expressed in sensory neurons and an important contributor to pain sensation (19;20). The β3 and β1 subunits differentially regulated heterologously expressed Na$^+_{1,7}$ channels. The preferential expression of β subunits in small (β1, β3) and large (β1, β2) neurons coupled with the isoform-specific β subunit regulation of Na$^+_{1,7}$ activation (β3) and inactivation (β1) predicts substantial differences in the TTX-S currents of DRG sensory neurons.

**EXPERIMENTAL PROCEDURES**

**Preparation of DRG neurons** - Neonatal (7 day old) Sprague Dawley rats (P7) were anaesthetized with isoflurane before decapitation and the dorsal root ganglia 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) 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% fetal bovine serum (Gibco Life Technologies), 2 mM glutamine, 2% penicillin-streptomycin (Gibco Life Technologies) and 50 ng/ml of nerve growth factor (Sigma-Aldrich). The ganglia were disrupted using fire-polished Pasteur pipettes and dissociated neurons were plated onto poly-lysine coated glass cover slips and placed into 35 mm dishes containing supplemented Leibovitz media. Neurons were suitable for single-cell harvesting and electrophysiology for up to 8 hours after plating. Animal protocols were approved by the Animal Care and Use Committee of Thomas Jefferson University.

**Single-cell RT-PCR** - Detailed methods for performing single-cell RT-PCR of dissociated DRG neurons were recently published (7). Small- (<25 μm) and large-diameter (>30 μm) DRG neurons are individually harvested by drawing them into a large bore pipette (30-50 μm diameter) containing sterile bath solution. The neurons are osmotically lysed by 10-fold dilution with sterile water and rapidly frozen. The mRNA present in the cell lysates was reverse-transcribed using random hexamer primers (Stratagene) in a standard 25 μl MMLV reverse transcription (RT) reaction (Fisher Scientific). Aliquots of the RT reaction (1-2 μl) were quantitatively analyzed using a SYBR green reaction cocktail on a MX30005P real-time PCR machine (Agilent Technologies). β-actin was quantitatively measured in each sample and used to normalize for differences in cellular mRNA expression. The absolute number of mRNA copies of each transcript was determined by comparing the threshold cycle (Ct) of the single-cell lysates with known cDNA standards assayed in parallel reactions. PCR primers are designed to span exon/intron borders to eliminate the detection of genomic DNA and concentrations (50-200 nM) optimized to achieve high amplification efficiency without the formation of primer dimers (Sigma-Proligo). The specificity of the real time detections were assessed using melting curve analysis and the identity of the amplified DNA determined by sequencing.

**Na$^+_{1,7}$ stable cell line** - Rat Na$^+_{1,7}$ cDNA was subcloned into the pcDNA3 expression vector (Invitrogen Corporation) and transfected into HEK293 cells using a standard calcium phosphate precipitation method (Invitrogen Corporation). After two weeks of selection for neomycin resistance (800 μg/ml) the remaining colonies were isolated and transferred to separate culture plates for expansion. Na$^+_{1,7}$ expression was verified using RT-PCR and electrophysiology to
measure Na\textsuperscript{+} currents. The HEK293 cell line stably expressing Na\textsubscript{v}1.7 were maintained using standard culture conditions in DMEM media supplemented with FBS (10%), 2 mM L-glutamine, 100 U/ml penicillin, 10 mg/ml streptomycin and 400 \mu g/ml neomycin (Gibco Life Technologies).

**Electrophysiology** - Macroscopic Na\textsuperscript{+} currents of HEK293 cells stably expressing the Na\textsubscript{v}1.7 channel were recorded using the whole-cell patch-clamp technique. The pipette solution contained (in mM) 5 NaCl, 135 CsF, 10 EGTA, and 10 HEPES (pH 7.4). The bath solution contained (in mM) 150 NaCl, 2 KCl, 1.5 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2} and 10 HEPES (pH 7.4). Patch electrodes were fashioned from 8161 borosilicate glass (Corning Incorporated) and coated with Sylgard (Dow-Corning Corporation) to minimize pipette capacitance. Recording pipettes had low access resistances (<1M\Omega) and the residual series resistance was 80% compensated. A correction for the liquid junction potential between the pipette and the bath solutions (-7 mV) was applied to the holding potential before the formation of gigaohm seals. After establishing the whole-cell configuration the cells were dialyzed for 10 minutes at room temperature (22°C) prior to recording Na\textsuperscript{+} currents. Voltage pulses were generated and currents recorded using pCLAMP and an Axopatch 200 amplifier (Molecular Devices). Whole cell currents were filtered at 5 kHz and digitized at 10 kHz with a Digidata 1440A (Molecular Devices).

Current-voltage relationships were obtained by plotting the current density (pA/pF) versus the test voltage. Normalized Na\textsuperscript{+} conductance (G\textsubscript{Na}) was calculated from the peak Na\textsuperscript{+} current (I\textsubscript{Na}) at each test potential (V): G\textsubscript{Na} = I\textsubscript{Na}/(V-E\textsubscript{Na}), where E\textsubscript{Na} is the measured Na\textsuperscript{+} ion reversal potential. The steady-state inactivation was determined by normalizing the peak Na\textsuperscript{+} current (I) measured after conditioning prepulses (-130 to -10, 500 ms) to the maximal Na\textsuperscript{+} current amplitude (I\textsubscript{max}) measured after prepulses to -140 mV and plotted against the conditioning voltage. The activation and steady-state inactivation were fitted to Boltzmann functions: G/G\textsubscript{max}(V/I\textsubscript{max}) = 1 /[1+exp (V\textsubscript{0.5} - V)/k\textsubscript{s}], where V\textsubscript{0.5} is the midpoint and k\textsubscript{s} the slope factor. The predicted window currents were calculated from the product of the activation and steady-state inactivation curves as described previously (21). Recovery from inactivation was determined using depolarizing prepulses (-30 mV/20 ms) before returning to −100 mV for variable intervals (0-1200 ms). Standard test pulses (-30 mV/20 ms) were used to assess availability. The recovery time course was fitted to the sum of two exponentials yielding estimates of the fast (\tau\textsubscript{f}) and slow (\tau\textsubscript{s}) time constants.

Rat \beta\textsubscript{11}, \beta\textsubscript{2} and \beta\textsubscript{3} subunits were cloned in our laboratory as described previously (22). The \beta\textsubscript{4} subunit was a gift from Dr. Lori Isom (University of Michigan). The Na\textsuperscript{+} channel \beta\textsubscript{1-4} subunits (piRES/CD8/\beta\textsubscript{1-4}) and CD8 cDNA were subcloned into the piRES vector (Clontech Laboratories). HEK293 cells stably expressing the Na\textsubscript{v}1.7 channel were transient transfected with piRES/CD8/\beta\textsubscript{1-4} cDNA using a calcium phosphate precipitation method (23). Prior to recording the cells were briefly incubated in PBS containing CD8 antibody-coated beads to identify cells expressing the CD8 antigen (Dynal, Lake Success, NY).

**\beta subunit chimeras** - The \beta\textsubscript{1}/\beta\textsubscript{2} chimeras (\beta\textsubscript{211}, \beta\textsubscript{221}, \beta\textsubscript{112}, \beta\textsubscript{11Δ}) were a gift from Dr. Thomas Zimmer (Friedrich Schiller University, Jena, Germany). The three subscripted numbers refer to the extracellular N-terminal, membrane-spanning and intracellular C-terminal domains. In this nomenclature the wild-type \beta\textsubscript{1} and \beta\textsubscript{2} subunits are designated \beta\textsubscript{111} and \beta\textsubscript{222} respectively. \beta\textsubscript{211} contains the extracellular domain of \beta2 and the membrane-spanning and intracellular domains of \beta1. \beta\textsubscript{221} incorporates the extracellular and membrane-spanning domains of \beta2 and the intracellular domain of \beta1. \beta\textsubscript{112} contains the extracellular and membrane-spanning domains of \beta1 and a deletion of the 41 amino acids from the intracellular C-terminal domain (Fig. 6. A). \beta\textsubscript{211}, \beta\textsubscript{221}, \beta\textsubscript{112}, and \beta\textsubscript{11Δ} were transfected to the piRES vector for expression in mammalian cells (piERS/CD8/\beta\textsubscript{211}, piERS/CD8/\beta\textsubscript{221}, piERS/CD8/\beta\textsubscript{112}, piERS/CD8/\beta\textsubscript{11Δ}) and transiently transfected into our Na\textsubscript{v}1.7 stable cell line.

**Immunoprecipitation and Western analysis** - Rat dorsal root ganglia (DRG) were harvested and immediately placed in ice cold Hank’s Balanced Salt Solution (HBSS). The ganglia were washed with ice cold HBSS and pelleted by low-speed centrifugation at 4°C. HBSS was replaced with ice cold lysis buffer (50mM Tris, 1.0mM EDTA, 1.0mM EGTA, 150mM NaCl, 1.0% Triton X 100) supplemented with protease inhibitors (Sigma-
Aldrich). The samples were homogenized on ice and centrifuged (15,000 rpm/20 mins) at 4°C. The supernatant was recovered and assayed for protein concentration using the Bradford method (Bio-Rad Corporation). Lysates (1 mg) were incubated overnight at 4°C in 1 ml of lysis buffer containing either 10 μg control mouse IgG or 10 μg mouse monoclonal N68/6 anti-Na,1.7 antibody (NeuroMab). The N68/6 anti-Na,1.7 antibodies do not cross-react with other Na+ channels isoforms or channel proteins extracted from adult rat brain. Protein G agarose resin (Thermo Scientific) was added (100 μl) and the lysates incubated 6 hours at 4°C before washing with ice-cold lysis buffer. Proteins were eluted from the protein G agarose by addition of 50 μl of 0.2M glycine buffer (pH 2.5). The pH was neutralized by adding 10 μl of Tris buffer (1M, pH 9.0), mixed with 3x sample buffer and separated on 12% SDS-PAGE gels. Proteins were transferred to Protran nitrocellulose membranes (Whatman International), blocked with 5% BSA, washed with Tris buffered saline with 0.1% Tween 20 (TBST) and incubated overnight with rabbit polyclonal SCN1B (Cell Applications), rabbit polyclonal SCN2B (Sigma-Aldrich) or rabbit polyclonal SCN3B (Abcam) antibodies in TBST containing 5% BSA. These commercial antibodies (SCN1B, SCN2B, SCN3B) are highly specific and do not display cross-reactivity with other members of the β subunit family. The membranes were incubated with HRP conjugated goat anti-rabbit secondary antibody (Thermo Scientific) for one hour at room temperature and labeled proteins were detected using chemiluminescence (Thermo Scientific). We routinely failed to observe Na,1.7 or β subunit precipitation from cell lysates preincubated with control IgG further supporting the specificity of the Na,1.7 pull downs. The low level expression of the β4 subunits in DRG neurons (Figure 1) combined with the poor quality of available β4 antibodies prevented detailed analysis of this protein.

**Immunocytochemistry** - Dissociated DRG neurons were plated onto poly-lysine coated glass cover slips and fixed in PBS containing 4% paraformaldehyde for 10 minutes. Cells were permeabilized with 0.1% Triton-X100 in PBS for 5 minutes before several washes with PBS. Non-specific antibody binding was reduced by incubating the cells with 5% BSA and 5% goat serum in TBST for 60 minutes. Permeabilized cells were incubated with mouse monoclonal anti-Na,1.7 antibody (NeuroMab), rabbit polyclonal SCN1B (Cell Applications), rabbit polyclonal SCN2B (Sigma-Aldrich) or rabbit polyclonal SCN3B (Abcam) antibodies (1:500 dilution) for 60 minutes before adding anti-mouse (Alexa Fluor 488) or anti-rabbit (Alexa Fluor 594) conjugated fluorescent secondary antibody for 60 minutes (Invitrogen Corporation). After several washes with PBS the cover slips were dried overnight and mounted onto glass slides with Mowiol 4.88 (Calbiochem). The slides were imaged on a Zeiss LSM 510 META confocal microscope equipped with FITC and Rhodamine filter sets at the Kimmel Cancer Center at Jefferson Medical College.

**RESULTS**

The expression of β subunits was investigated in acutely dissociated DRG sensory neurons isolated from 7 day old neonatal rats. Neurons were individually harvested and the mRNA present in the cell lysates quantitatively measured (mRNA copies/neuron) using real-time PCR. Figure 1 compares the expression of the β subunit transcripts in small- (<25 μm) and large-diameter (>30 μm) DRG neurons. The data indicate that small neurons preferentially express the β2 and β3 isoforms (2000-4000 copies/neuron). Although β1 was also detected in these neurons the mRNA copy number was 5-fold lower (<400 copies/neuron). This contrasts with large-diameter neurons that highly expressed β1 and β3 mRNA (~4500 copies/neuron) while β3 was present at lower levels (<2000 copies/neuron). The β4 subunit was expressed at comparatively low levels in both the small (<500 copies/neuron) and large (<2000 copies/neuron) neurons. The data indicate that small (β2, β3) and large (β1, β2) DRG neurons express different complements of auxiliary β subunits.

To investigate the relationship between Na,1.7 and β subunits the mRNA encoding for these subunits were quantitatively measured in small and large DRG neurons. Figure 2 plots the number of Na,1.7 mRNA copies versus the β subunit mRNA measured from the same neurons. The data were statistically evaluated using Pearson produce-moment correlation analysis to determine the strength of mRNA co-expression in these neurons. The Na,1.7-β2 and Na,1.7-β3 mRNAs were found to be significantly correlated with
Potential Na\textsubscript{v}1.7-β subunit interactions were further investigated using immunocytochemistry. Figure 4 shows the confocal imaging of small neurons labeled with Na\textsubscript{v}1.7 and β-specific antibodies. The cytoplasm of these neurons displayed diffuse labeling for the Na\textsubscript{v}1.7, β1 and β2 subunits. Merged images revealed some overlap of Na\textsubscript{v}1.7 with β1 and β2 subunits, predominately within the intracellular compartment. By contrast, the majority of the β3 immunofluorescence was localized along the cell periphery consistent with the labeling of membrane bound proteins. The merged images display considerable overlap of Na\textsubscript{v}1.7 and β3 around the cell periphery consistent with the co-localization of these proteins near the plasma membrane.

Initial attempts to investigate the β subunit regulation of endogenous Na\textsubscript{v}1.7 channels in dissociated DRG neurons were complicated by the variable expression of Na\textsubscript{v}1.7 and β subunits and the presence of multiple overlapping components of TTX-S Na\textsuperscript{+} current in these neurons. We therefore conducted heterologous expression studies to further investigate the β subunit regulation of Na\textsubscript{v}1.7 channels. HEK293 cells stably expressing Na\textsubscript{v}1.7 were transiently transfected with β subunits. Figure 5 shows examples of whole-cell Na\textsuperscript{+} currents recorded from cells expressing Na\textsubscript{v}1.7 alone or with co-expressed β1 or β3 subunits. In the absence of β subunits the Na\textsubscript{v}1.7 channels produced rapidly gating Na\textsuperscript{+} current. Co-expressing β subunits (β1-β2) had no effect on the current kinetics or peak Na\textsuperscript{+} current amplitudes.

To investigate potential changes in voltage-dependent gating the Na\textsuperscript{+} conductance was calculated from the peak currents and plotted versus the test voltage (Figure 6A). Co-expressing the β1 subunit produced a significant hyperpolarizing shift (-9 mV) in Na\textsubscript{v}1.7 activation. Steady-state inactivation was determined using 500 ms prepulses to voltages between −130 and −5 mV. β1 induced a depolarizing shift (+5 mV) in the midpoint of Na\textsubscript{v}1.7 inactivation (Figure 6A). By contrast, co-expressing the β2 or β4 subunits did not alter the activation or the steady-state inactivation of the channels.

Recovery from inactivation was determined by applying depolarizing prepulses (−30 ms/20 mV) before returning to −100 mV for varying intervals (0-1200 ms). The recovery time course of Na\textsubscript{v}1.7 channels was biexponential with fast (τ\textsubscript{f}) and slow
(\(\tau_c\)) time constants of 26 ms and 153 ms respectively (Figure 6B). Co-expressing \(\beta_1\) significantly reduced both \(\tau_c\) (14 ms) and \(\tau_i\) (67 ms) consistent with more rapid recovery from inactivation (Figure 6B). The remaining \(\beta\) subunits (\(\beta_2-\beta_4\)) had no effect on recovery from inactivation (Table 1).

The overlap of activation and steady-state inactivation of Na\(^+\) channels defines a range of voltages (i.e. window) where Na\(^+\) channels can be partially activated but are not fully inactivated. Na\(^+\) channels within this hyperpolarized range of voltages may become persistently activated resulting in inward Na\(^+\) currents that could potentially depolarize the resting membrane potential and increase neuronal excitability. \(\beta\)-induced increases in the overlap of Na\(^+\) channel activation and inactivation tend to expand this window and consequently the fraction of persistently activated channels. The \(\beta_1\) subunit produced a +5 mV depolarizing shift in steady-state inactivation while \(\beta_3\) produced a -9 mV shift in Na\(^+\)1.7 activation (Table 1) that could potentially increase the window currents. Figure 6C shows the predicted window currents of Na\(^+\)1.7 channels co-expressed with either the \(\beta_1\) or \(\beta_3\) subunits. Despite acting by different mechanisms the \(\beta_1\) and \(\beta_3\) subunits produce similar 2-3 fold increases in the Na\(^+\)1.7 window current.

To gain a better understanding of the mechanism of \(\beta\) subunit regulation chimeras were generated by exchanging the structural domains of the \(\beta_1\) subunit that shifted steady-state inactivation and accelerated recovery from inactivation with the homologous domains of the \(\beta_2\) subunit that had no effect on Na\(^+\)1.7 gating (Table 1). The extracellular N-terminal, intracellular C-terminal and membrane-spanning domains of \(\beta_1\) were systematically replaced with those of \(\beta_2\) and transiently expressed in HEK293 cells stably expressing Na\(^+\)1.7 channels. Chimeras that retained the extracellular N-terminal domain of \(\beta_1\) (\(\beta_{112}, \beta_{11\Delta}\)) fully recapitulated the hyperpolarizing shift in steady-state inactivation and faster recovery observed with the wild-type \(\beta_1\) subunit (Table 1). Conversely, substitutions that replaced the N-terminus of \(\beta_1\) (\(\beta_{211}, \beta_{221}\)) completely abolished Na\(^+\)1.7 regulation. C-terminal deletions of the \(\beta_1\) subunit (\(\beta_{11\Delta}\)) retained full activity indicating that intracellular domain is not essential. The data indicate that the extracellular N-terminal domain of \(\beta_1\) is critical for the functional regulation of Na\(^+\)1.7 channels.

**DISCUSSION**

The goal of these studies was to investigate the expression of auxiliary \(\beta\) subunits in DRG neurons and to characterize the \(\beta\) subunit regulation of Na\(^+\)1.7, a TTX-S Na\(^+\) channel widely expressed in sensory neurons. Single-cell analysis found that \(\beta\) subunit mRNAs were differentially expressed in small (\(\beta_2, \beta_3\)) and large (\(\beta_1, \beta_2\)) DRG neurons (Figure 1). Comparisons of Na\(^+\)1.7, \(\beta_2\) and \(\beta_3\) mRNA measured from individual small neurons found that the expression of these subunits were significantly correlated indicating that these transcripts are co-expressed in the same neurons (Figure 2). By contrast, the Na\(^+\)1.7 mRNA of large neurons was found to be significantly correlated with the \(\beta_1\) and \(\beta_2\) subunits. These data indicate that the Na\(^+\)1.7 channels present in small and large DRG neurons are co-expressed with different complements of auxiliary \(\beta\) subunits.

Interactions between Na\(^+\)1.7 and \(\beta\) subunits were further explored using co-immunoprecipitation of Na\(^+\)1.7 channels. Na\(^+\)1.7 co-precipitated with the \(\beta_1, \beta_2\) and \(\beta_3\) subunits indicating that these subunits form stable complexes in vivo (Figure 3). Despite supporting a direct physical interaction between Na\(^+\)1.7 and the \(\beta_1-\beta_3\) subunits the neurons in which these interactions occurred (i.e. small vs. large) is impossible to ascertain using immunoprecipitation techniques. However, immunofluorescent imaging showed that Na\(^+\)1.7 and \(\beta_3\) co-localized near the periphery of the small DRG neurons (Figure 4). Although \(\beta_2\) subunits are also highly expressed in small neurons they failed to display obvious co-localization with Na\(^+\)1.7 channels. The combination of Na\(^+\)1.7-\(\beta_3\) mRNA correlation (Figure 2), co-immunoprecipitation (Figure 3) and co-localization near at the plasma membrane (Figure 4) support the idea that \(\beta_3\) subunits partner with Na\(^+\)1.7 channels. While these data do not preclude Na\(^+\)1.7 interaction with other \(\beta\) subunits it suggests an important contribution of Na\(^+\)1.7-\(\beta_3\) channels to the TTX-S Na\(^+\) currents of small DRG neurons.

Previous studies of \(\beta\) subunit regulation of heterologously expressed Na\(^+\)1.7 channels have produced conflicting data. Initial studies of Na\(^+\)1.7 channels expressed in *Xenopus* oocytes indicated that the \(\beta_1\) and \(\beta_2\) subunits failed to alter the
expression or gating properties of Na\(_{1.7}\) suggesting that these channels may be not regulated by these auxiliary subunits (27;28). Subsequent work, also in oocytes, found that co-expressing β\(_1\) accelerated inactivation and recovery kinetics and produced a hyperpolarizing shift in Na\(_{1.7}\) activation (29). The regulation of Na\(_{1.7}\) channels by the β\(_3\) and β\(_4\) subunits has not been investigated.

In this study HEK293 cells stably expressing Na\(_{1.7}\) channels were employed to further investigate the functional consequences of Na\(_{1.7}\)-β interactions. Co-expressing β subunits (β\(_1\);β\(_4\)) did not alter the peak Na\(^+\) current densities or Na\(_{1.7}\) current kinetics. However, β\(_1\) produced a depolarizing shift in steady-state inactivation and faster recovery from inactivation (Table 1). At voltages near the resting membrane potentials of DRG neurons (~60 mV), depolarizing shifts in inactivation would tend to increase the fraction of Na\(_{1.7}\) channels available to open in response to depolarization. Similar increases in availability along with the associated increase in Na\(^+\) current density are well known to reduce the threshold for initiating action potentials (30-32). The rate of Na\(^+\) channel recovery from inactivation is an important determinant of the absolute and relative refractory periods of action potentials. The faster recovery of Na\(_{1.7}\)-β\(_1\) channels predicts rapid repriming at hyperpolarized voltages that may reduce the duration of the refractory periods thereby enabling increased firing frequency in large-diameter neurons highly expressing the Na\(_{1.7}\)-β\(_1\) combination.

The β\(_3\) subunit produced a -9 mV shift in Na\(_{1.7}\) activation causing the channels to open at more hyperpolarized voltages (Table 1). Such shifts in activation and the accompanying increase in Na\(^+\) current at more hyperpolarized voltages are predicted to increase neuronal excitability and could potentially reduce the threshold for firing action potentials in small-diameter neurons. This mechanism is consistent with studies showing that Na\(^+\) channels with low activation thresholds are critical determinants of action potential initiation at the axon initial segment (33;34).

Na\(_{1.7}\)-β subunit interactions that induce hyperpolarizing shifts in activation (β\(_3\)) or depolarizing shifts in inactivation (β\(_1\)) tend to increase the overlap of activation and inactivation gating (Figure 6C). At voltages within this overlap region Na\(^+\) channels are partially activated but not fully inactivated increasing the potential of persistent window currents (35). At -50 mV, the peak window current probability predicts that a small percentage (0.1%) of Na\(_{1.7}\) channels will be persistently activated. Co-expressing the β\(_1\) or β\(_3\) subunits increased the probability of persistent activation by 2-3 fold. Persistent activation of Na\(_{1.7}\)-β\(_1\) and Na\(_{1.7}\)-β\(_3\) channels and the resulting inward Na\(^+\) current at resting membrane potentials could depolarize the neuron leading to increased excitability of DRG neurons. Similar mechanisms are believed to underlie the increased excitability of sensory neurons harboring inherited human pain disorder mutations that produce shifts in Na\(_{1.7}\) activation and inactivation of similar polarity and magnitude as those observed for the Na\(_{1.7}\)-β\(_1\) and Na\(_{1.7}\)-β\(_3\) channels (36-38).

Previous studies have employed chimera, deletion analysis and mutations to define the structural domains of β subunits that are critical for Na\(^+\) channel regulation (21;39-42). The findings indicate that the extracellular N-terminal domain of β\(_1\) is essential for the functional regulation of neuronal and skeletal muscle Na\(^+\) channels. This contrasts with the β\(_1\) regulation of cardiac Na\(^+\) channels, where the membrane-spanning domain was found to be critical for the increased expression and accelerated recovery of Na\(_{1.5}\) channels (43). These data imply that different structural domains and therefore different molecular interactions are responsible for β\(_1\) regulation of neuronal and cardiac Na\(^+\) channels.

β\(_1\) mRNA is highly expressed in large DRG neurons (Figure 1) where it is significantly correlated with Na\(_{1.7}\) indicating that these subunits are co-expressed in the same population of large-diameter neurons (Figure 2). β subunit chimeras were employed to identify the structural domains of β\(_1\) required to produce the observed depolarizing shift in steady-state inactivation and accelerated recovery of Na\(_{1.7}\) channels (Table 1). Chimeras incorporating the extracellular N-terminal domain of β\(_1\) (β\(_{112}\)) retained the shift in inactivation and faster recovery while replacing the extracellular domain (β\(_{211}\)) completely eliminated these effects. These data indicate that the N-terminal domain of β\(_1\) subunit is required for Na\(_{1.7}\) regulation. β\(_1\) subunits with a truncated C-terminus (β\(_{113}\)) retained full functional regulation indicating that the intracellular domain is non-essential. Interactions between the N-terminus of β\(_1\) and extracellular loops of Na\(_{1.7}\) may be important for the functional regulation of these
channels similar to what has been previously described for other neuronal Na\(^+\) channels (40;41). Recent work employed a similar approach to investigate the \(\beta_1\) regulation of Na\(_{1.8}\), a TTX-R channel that produces the majority of the inward Na\(^+\) current in small-diameter DRG neurons (21). Substitution of the extracellular N-terminal domain of \(\beta_1\) had no effect on the expression or gating properties of Na\(_{1.8}\) channels. Rather the intracellular C-terminal domain of \(\beta_1\) was found to be the critical determinant of Na\(_{1.8}\) regulation. These data indicate that the N- and C-terminals of the \(\beta_1\) subunit differentially regulate the gating properties of Na\(_{1.7}\) and Na\(_{1.8}\) channels.

Much of what is currently known about \(\beta\) subunit expression in the DRG is derived from immunocytochemistry and in situ hybridization (4;12;15-18;44). These studies indicate that all four isoforms of \(\beta\) subunits (\(\beta_1\)-\(\beta_4\)) are present in the DRG and that these subunits are differentially expressed in subpopulations of sensory neurons (12;15). \(\beta_3\) subunits are prominently expressed in small- and medium-sized neurons while \(\beta_1\) and \(\beta_4\) are preferentially expressed in large neurons (4;16;17). \(\beta_2\) appears to be widely expressed in the DRG and does not show a clear preference for neuronal size (15;45). These findings are in good agreement with our single-cell analysis of gene expression and are consistent with the conclusion that \(\beta\) subunits are differentially expressed in subpopulations of DRG neurons. Unfortunately, histological approaches do not provide quantitative assessments of \(\beta\) subunits expression levels or insight into the functional regulation Na\(^+\) channels by \(\beta\) subunits. Our data indicate that the differential expression of \(\beta\) subunits in DRG neurons combined with isoform-specific \(\beta\) subunit regulation of Na\(_{1.7}\) activation (\(\beta_3\)) and inactivation (\(\beta_1\)) predicts substantial differences in the predominant TTX-S Na\(^+\) currents of small and large sensory neurons.

Previous work investigated the role of the \(\beta_1\) and \(\beta_2\) subunits in sensory neurons using SCN1B and SCN2B null mice (45;46). Whole-cell recordings from DRG neurons isolated from the \(\beta_1\) knockouts revealed small changes in the amplitudes and gating properties of TTX-S and TTX-R Na\(^+\) currents (46). The relatively subtle effects of the SCN1B knockout on DRG Na\(^+\) currents coupled with the low level expression of \(\beta_1\) subunits in small-diameter sensory neurons suggests that these subunits may not be important regulators of the Na\(^+\) channels expressed in nociceptors. Neurons from the SCN2B null mice displayed reductions in TTX-S Na\(^+\) current amplitude, Na\(^+\) channel mRNA and protein (46). Although the underlying mechanism is unclear the SCN2B knockout appears to reduce TTX-S Na\(^+\) currents by decreasing Na\(^+\) channel mRNA and protein expression. Based on the comparison of Na\(^+\) currents recorded from control and SCN2B null mice the \(\beta_2\) subunits were proposed to increase Na\(^+\) channel expression (Na\(_{1.1}\), Na\(_{1.6}\), Na\(_{1.7}\)), produce hyperpolarizing shifts in activation and accelerate the kinetics of the endogenous TTX-S Na\(^+\) currents (46). These effects were not recapitulated in our heterologous expression studies of Na\(_{1.7}\)-\(\beta_2\) channels where no changes in Na\(^+\) current density, voltage-dependence or current kinetics were observed. Rather our findings are consistent with previous work showing that the \(\beta_2\) subunit has no effect on the expression or gating properties of the Na\(_{1.3}\), Na\(_{1.6}\) or Na\(_{1.8}\) channels (47;48). The reasons for the apparent discrepancy between in vivo knockdown and heterologous expression studies are not known but may reflect contributions by endogenous regulatory pathways that are specific to the DRG or the compensatory upregulation of other \(\beta\) subunits in the sensory neurons of SCN2B null mice.

References

Differential expression of $\beta$ subunits in DRG neurons


Differential expression of β subunits in DRG neurons


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Figure Legends

**Figure 1. Single-cell analysis of β subunit mRNA.** Small- (<25 μm) and large-diameter (>30 μm) DRG neurons were individually harvested, the mRNA present in the cell lysates reverse transcribed and quantitatively measured using real-time PCR. The data are expressed as the number of mRNA copies present in each neuron. The data are the means and SEM of 74 and 21 small and large neurons respectively.

**Figure 2. Correlation of Na\textsubscript{v}1.7 and β subunit mRNA expression in small and large DRG neurons.** The mRNA (copies/neuron) of Na\textsubscript{v}1.7 and β subunits were measured from the same populations of small-diameter (<25 μm) and large-diameter (>30 μm) neurons. A-D. Plots of Na\textsubscript{v}1.7 mRNA versus β\textsubscript{1} (Panel A), β\textsubscript{2} (Panel B), β\textsubscript{3} (Panel C) and β\textsubscript{4} (Panel D). The straight lines are simple linear regressions. The data represent the means and SEM of mRNA measurements from 29 small and 21 large DRG neurons.

**Figure 3. Co-immunoprecipitation of Na\textsubscript{v}1.7 and β subunits.** A. DRG homogenates were separated on SDS-Page gels, transferred to nitrocellulose membranes and probed with Na\textsubscript{v}1.7-specific antibodies. B-D. Na\textsubscript{v}1.7 channel complexes were immunoprecipitated from DRG lysates, separated on SDS-page gels and probed with antibodies specific for β\textsubscript{1} (Panel B), β\textsubscript{2} (Panel C) or β\textsubscript{3} (Panel D). Tick marks indicate the position of molecular weight markers in kilodaltons (kDa).

**Figure 4. Imaging of Na\textsubscript{v}1.7 and β subunits in small DRG neurons.** Small-diameter (<25 μm) DRG neurons were immunolabeled with Na\textsubscript{v}1.7- and β– specific (β\textsubscript{1}-β\textsubscript{3}) antibodies and reacted with fluorochrome-conjugated secondary antibodies before confocal imaging. The left panels show the Na\textsubscript{v}1.7 immunostaining, the middle panels are the β subunit staining and the right panels are the merged images.

**Figure 5. β subunit regulation of heterologously expressed Na\textsubscript{v}1.7 channels.** Whole-cell Na\textsuperscript{+} currents of HEK293 cells stably expressing the Na\textsubscript{v}1.7 channels. Currents were elicited by depolarizing voltage pulses between -90 and +50 mV from a holding potential of -120 mV. A-C. Representative Na currents of Na\textsubscript{v}1.7 channels expressed alone (panel A) or co-expressed with β\textsubscript{1} (panel B) or β\textsubscript{3} subunits (panel C). D. Plot of the peak current density (pA/pF) of Na\textsubscript{v}1.7 channels alone or with co-expressed β subunits (β\textsubscript{1}-β\textsubscript{4}). Data are the means and SEM of 13 (Na\textsubscript{v}1.7), 26 (β\textsubscript{1}), 9 (β\textsubscript{2}), 18 (β\textsubscript{3}) and 8 (β\textsubscript{4}) determinations.

**Figure 6. β subunits shift activation and inactivation of Na\textsubscript{v}1.7 channels.** A. The normalized conductance was determined from the peak Na\textsuperscript{+} currents and plotted versus the test potential. Also plotted in the steady-state inactivation obtained using 500 ms prepulses to voltages between -130 and -5 mV. The smooth curves are fits of the activation and inactivation data to Boltzmann functions with the parameters listed in Table 1. Data are the means and SEM of 14 (Na\textsubscript{v}1.7), 26 (β\textsubscript{1}), 9 (β\textsubscript{2}), 21 (β\textsubscript{3}) and 8 (β\textsubscript{4}) determinations. B. Na channels were inactivated by a brief depolarization (-30 mV/20 ms) and the recovery time course (0-1200 ms) measured at –100 mV. The smooth curves are biexponential curve fits with fast and slow time constants listed in Table 1. Data are the means and SEM of 15 (Na\textsubscript{v}1.7), 22 (β\textsubscript{1}), 10 (β\textsubscript{2}), 17 (β\textsubscript{4}) and 8 (β\textsubscript{4}) determinations. C. Window current probabilities predicted from the activation and steady-state inactivation of the Na\textsubscript{v}1.7 channels expressed alone or with either the β\textsubscript{1} or β\textsubscript{3} subunits.
Table 1. β subunit regulation of Nav1.7 gating

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<th>Inactivation</th>
<th>Recovery</th>
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The parameters were obtained from curve fits of Nav1.7 activation, inactivation and recovery from inactivation (Figure 6). The data were tested for significant differences using ANOVA (p<0.001) followed by post-hoc Dunnett’s test at a significance level of p<0.05. For Dunnett’s test the effects of beta subunits were compared to values measured for Nav1.7 channels expressed alone. Data are the means and SEM of between 8 and 30 experiments.
Differential expression of β subunits in DRG neurons

Figure 1

Single-cell analysis of β subunit mRNA. Small (<25 μm) and large-diameter (>30 μm) DRG neurons were individually harvested, the mRNA present in the cell lysates reverse transcribed and quantitatively measured using real-time PCR. The data are expressed as the number of mRNA copies present in each neuron. The data are the means and SEM of 74 and 21 small and large neurons respectively.
Differential expression of β subunits in DRG neurons

Figure 2

A

\[ \beta_1 \text{ mRNA (copies/neuron)} \]

\[ \text{Nav1.7 mRNA (copies/neuron)} \]

<25 μm  
△ >30 μm

B

\[ \beta_2 \text{ mRNA (copies/neuron)} \]

\[ \text{Nav1.7 mRNA (copies/neuron)} \]

C

\[ \beta_3 \text{ mRNA (copies/neuron)} \]

\[ \text{Nav1.7 mRNA (copies/neuron)} \]

D

\[ \beta_4 \text{ mRNA (copies/neuron)} \]

\[ \text{Nav1.7 mRNA (copies/neuron)} \]
Differential expression of β subunits in DRG neurons

Co-immunoprecipitation of Nav1.7 and β subunits.

A. DRG homogenates were separated on SDS-Page gels, transferred to nitrocellulose membranes and probed with Nav1.7-specific antibodies.

B-D. Nav1.7 channel complexes were immunoprecipitated from DRG lysates, separated on SDS-page gels and probed with antibodies specific for β1 (Panel B), β2 (Panel C) or β3 (Panel D). Tick marks indicate the position of molecular weight markers in kilodaltons (kDa).

Figure 3
Imaging of Nav\textsubscript{1.7} and $\beta$ subunits in small DRG neurons. Small-diameter (<25 μm) DRG neurons were immunolabeled with Nav\textsubscript{1.7} and $\beta$–specific ($\beta_1$–$\beta_3$) antibodies and reacted with fluorochrome-conjugated secondary antibodies before confocal imaging (see Methods). The left panels show the Nav\textsubscript{1.7} immunostaining, the middle panels are the $\beta$ subunit staining and the right panels are the merged images.

**Figure 4**
Differential expression of β subunits in DRG neurons

Figure 5

A. Nav1.7

B. Nav1.7-β1

C. Nav1.7-β3

D. Plot of the peak current density (pA/pF) of Nav1.7 channels alone or with co-expressed β subunits (β1-β4). Data are the means and SEM of 13 (Nav1.7), 26 (β1), 9 (β2), 18 (β3) and 8 (β4) determinations.
Differential expression of β subunits in DRG neurons

Figure 6

A

B

C

Differential expression of β subunits in DRG neurons

Figure 6

A

B

C

Differential expression of β subunits in DRG neurons
Differential expression of sodium channel β subunits in dorsal root ganglion sensory neurons

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