α- and β-subunit composition of voltage-gated sodium channels investigated with μ-conotoxins and the recently discovered μO§-conotoxin GVIIJ

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Wilson MJ, Zhang MM, Gajewiak J, Azam L, Rivier JE, Olivera BM, Yoshikami D. α- And β-subunit composition of voltage-gated sodium channels investigated with μ-conotoxins and the recently discovered μO§-conotoxin GVIIJ. J Neurophysiol 113: 2289–2301, 2015. First published January 28, 2015; doi:10.1152/jn.01004.2014.—We investigated the identities of the isoforms of the α (NaV1.1–1.9) and β (NaVβ1–β3) subunits of voltage-gated sodium channels, including those responsible for action potentials in rodent sciatic nerves. To examine α-subunits, we used seven μ-conotoxins, which target site 1 of the channel. With the use of exogenously expressed channels, we show that two of the μ-conotoxins, μ-BuIIIB and μ-SxIII A, are 50-fold more potent in blocking NaV1.6 from mouse than that from rat. Furthermore, we observed that μ-BuIIIB and μ-SxIII A are potent blockers of large, myelinated A-fiber compounds action potentials (A-CAPs) [but not small, unmyelinated C-fiber CAPs (C-CAPs)] in the sciatic nerve of the mouse (unlike A-CAPs of the rat, previously shown to be insensitive to these toxins). To investigate β-subunits, we used two synthetic derivatives of the recently discovered μO§-conotoxin GVIIJ that define site 8 of the channel, as previously characterized with cloned rat NaV1.1 and NaVβ1–β3-subunits expressed in Xenopus laevis oocytes, where it was shown that μO§-GVIIJ is a potent inhibitor of several NaV1.1 isoforms and that coexpression of NaVβ2 or β4 (but not NaVβ1 or β3) totally protects against block by μO§-GVIIJ. We report here the effects of μO§-GVIIJ on J) sodium currents of mouse NaV1.6 coexpressed with various combinations of NaVβ-subunits in oocytes; 2) A- and C-CAPs of mouse and rat sciatic nerves; and J) sodium currents of small and large neurons dissociated from rat dorsal root ganglia. Our overall results lead us to conclude that action potentials in A-fibers of the rodent sciatic nerve are mediated primarily by NaV1.6 associated with NaVβ2 or NaVβ4.

dorsal root ganglion neuron; conotoxin; NaV1.6; NaVβ-subunit; sciatic nerve action potential

VOLTAGE-GATED SODIUM CHANNELS (VGSCs) are responsible for the upshot of action potentials. Each VGSC consists of an α-subunit (of which there are nine isoforms, NaV1.1–1.9) and one or more NaVβ-subunits (of which there are four isoforms, NaVβ1–β4), likely either an NaVβ1- or NaVβ3-subunit and either an NaVβ2- or NaVβ4-subunit to form a ternary, heterotrimeric αββ complex (Calhoun and Isom 2014; Catterall 2012). The α-subunit has some 2,000 amino acid (AA) residues and consists of four homologous domains (DI–DIV), each with six membrane-spanning segments (S1–S6). Each domain has a voltage-sensor module (comprised of S1–S4) and a pore module (comprised of S5–S6, with their connecting re-entrant loop consisting of short segments SS5 and SS6), and the four domains are radially arranged, with each pore module contributing to a central, ion-conducting pore (Catterall 2014). The β-subunit has some 200 AA residues and forms a single transmembrane-spanning segment that separates a large, extracellular domain from a smaller, intracellular C-terminal tail. The NaVβ1- or NaVβ3-subunit is noncovalently associated with the α-subunit, whereas the NaVβ2- or NaVβ4-subunit is covalently coupled to the α-subunit via a disulfide bond located on the extracellular aspect of the subunits (Calhoun and Isom 2014; Catterall 2012; Chen et al. 2012; Gilchrist et al. 2013; Yu et al. 2003). A given neuron can have more than one single isoform of an α-subunit, and we are developing approaches to identify the functional contributions of each isoform through the use of conotoxins (Wilson et al. 2011a; Zhang et al. 2013b).

Five families of conotoxins that target sodium channels have been identified thus far. Three families consist of peptides that are antagonists: μ-conotoxins, which are pore blockers, like TTX, and compete with TTX in binding to site 1 (Cestèle and Catterall 2000; Cruz et al. 1985; Zhang et al. 2009, 2010a); μO-conotoxins, which are gating modifiers that inhibit channel activation by interacting with site 4, the extracellular loop connecting S3 and S4 of DII (Heinemann and Leipold 2007; Leipold et al. 2007); and μO§-conotoxins, which bind to site 8, centered on a Cys residue between S5 and S6S of DII [specifically, C910 in the case of rat Nav1.2 (rNav1.2)] but whose mechanism of block remains to be established (Gajewiak et al. 2014). The other two conotoxin families consist of peptides that are VGSC agonists: δ-conotoxins, which inhibit channel inactivation by binding to site 6 at the extracellular loop between S3 and S4 of DIV (Leipold et al. 2005) and ε-conotoxins, which promote channel activation but whose site of action on VGSCs remains to be established (Fiedler et al. 2008). [For a recent review, see Stevens et al. (2011).] These toxins possess varying degrees of NaV1.2 specificities when examined with Xenopus laevis oocytes expressing cloned NaV1.2.

A compound action potential (CAP) consists of extracellularly recorded action potentials of a population of axons (or fibers). A- and C-CAPs are conducted by large, myelinated A-fibers and small, unmyelinated C-fibers, respectively; thus A-CAPs have faster conduction velocities than C-CAPs, and their waveforms are readily distinguishable by their latencies. We used a panel of μ-conotoxins to conclude that A- and C-CAPs in the rat sciatic nerve are mediated principally by NaV1.6 and -1.7, respectively (Wilson et al. 2011a). We also used μ-conotoxins to show that NaV1.1, -1.6, and -1.7 could...
account for all of the TTX-sensitive voltage-gated sodium currents (INa) in cell bodies of acutely dissociated rat dorsal root ganglia (DRG) neurons; furthermore, the levels of functional NaV1.1s in large neurons were NaV1.1 ≤ NaV1.7 ≤ NaV1.6. In a class of small neurons, whose INa was >50% sensitive to TTX, the levels were NaV1.1 ≤ NaV1.6 < NaV1.7 (Zhang et al. 2013b).

In contrast to NaV1.6 from mouse (mNaV1.6), much less has been reported concerning the functional and pharmacological properties of NaV1.6 from rat (rNaV1.6) since its initial description (Dietrich et al. 1998) until relatively recently (Gajewiak et al. 2014; He and Soderlund 2014; Tan et al. 2011; Tan and Soderlund 2011; Zhang et al. 2013a). While comparing the abilities of various μ-conotoxins in blocking mNaV1.6 expressed in oocytes and A-fibers of the rat sciatic nerve, we encountered an apparent discrepancy; that is, at the time of those experiments, only an mNaV1.6 clone was available to us, and we noted that two of the μ-conopeptides tested, μ-SxIIIα and μ-BullIB, which were potent in blocking mNaV1.6 expressed in oocytes, were unable to block A-CAPs in the rat sciatic nerve, unlike five other μ-conotoxins (μ-SmlIIIα, μ-KIIIα, μ-SIIIα, μ-GIIIα, and μ-PIIIα) that readily blocked both rat A-CAPs and mNaV1.6 (Wilson et al. 2011a). It might be noted that all five of the latter μ-conotoxins blocked mNaV1.6 with submicromolar affinities (Wilson et al. 2011a), and two of them, μ-SmlIIIα and μ-PIIIα, were tested on rNaV1.6, where they also blocked with submicromolar affinities (Zhang et al. 2013a). To reconcile the discrepancy, we suggested that the two μ-conotoxins, μ-SxIIIα and μ-BullIB, might be better able to block mNaV1.6 than rNaV1.6 (Wilson et al. 2011a). With the use of our recently acquired clone of rNaV1.6 (Zhang et al. 2013a), we show here that this is indeed the case.

The affinities of μ-conotoxins for rNaV1.6s can be affected by coexpression of NaVβ-subunits (Zhang et al. 2013a); thus we examined how the affinity of μ-SxIIIα for mNaV1.6 is affected by coexpression with various combinations of rNaVβ-subunits in oocytes (note, only rat clones of NaVβ-subunits are presently available to us). Additionally, we tested μ-SxIIIα and μ-BullIB along with five other μ-conotoxins on A- and C-CAPs of the mouse sciatic nerve to help identify likely NaV1-isoforms responsible for the propagation of action potentials in mouse A- and C-fibers.

We also tested two closely related synthetic derivatives of the recently discovered μO§-conotoxin GVI11, namely, μO§-GVI11SSG and μO§-GVI11SSC, where the subscripts refer, respectively, to cysteinylated and glutathionylated analogs that share the same peptide backbone (see MATERIALS AND METHODS). μO§-GVI11SSG was previously tested on oocytes expressing rNaV1.1–rNaV1.8, and it potently blocked all except NaV1.5 and -1.8, which were blocked poorly and not at all, respectively (Gajewiak et al. 2014). Except for a small (approximately threefold) difference in association rate constant (kə) (Gajewiak et al. 2014), both derivatives behave similarly in all functional tests performed thus far, including those reported here. For historical reasons, the early experiments were largely performed with μO§-GVI11SSG, whereas more recent experiments involved μO§-GVI11SSC, because its structure turned out to resemble more closely that of the native peptide (Gajewiak et al. 2014) (see MATERIALS AND METHODS). For brevity, the condensed term μO§-GVI11SSG will be used when referring to both peptides.

The binding site of μO§-GVI11SSG on the channel, site 8, is spatially distinct from those of site 1 (where μ-conotoxins bind) and site 4 (where μO-conotoxins bind), insofar as electrophysiological tests reveal that neither the μ-conotoxin derivative μ-KIIIα[K7A] nor μO-conotoxin MrVIB interferes with the block of rNaV1.2 by μO§-GVI11SSG (Gajewiak et al. 2014).

A novel feature of μO§-GVI11SSG, originally observed with μO§-GVI11SSG, is that coexpression in oocytes of rNaV1.1s with either NaVβ2 or -β4 protects the channel against block by the peptide (Gajewiak et al. 2014). We demonstrate here that this is also true for mNaV1.6; specifically, coexpression with NaVβ2 or -β4 (but not NaVβ1 or -β3) protects the channel against block by μO§-GVI11SSG. We also show that unlike members of all other conopeptide families that target VGSCs, μO§-GVI11SSG had no effect on A- and C-CAPs of rat and mouse sciatic nerves. Finally, we examined the ability of μO§-GVI11SSG to block INa mediated by VGSCs endogenously expressed in acutely dissociated neurons of rat DRG, and observed that INa of small, but not large, neurons could be blocked by the peptide.

MATERIALS AND METHODS
Toxins. μ-Conotoxins were synthesized as described previously (Wilson et al. 2011a). TTX was obtained from Alomone Labs (Jerusalem, Israel). δ-Conotoxins PVIA (δ-PVIA) was synthesized as described previously (Bulaj et al. 2001).

The sequence of μO§-GVI11 is as follows: GWCGDOGATCGKLRLYCSCGFCDCTKTKDDKSSA, where the seven Cys residues are in boldface. O represents hydroxyproline, the underlined C is Cys24, and the caret signifies a free carboxyl terminus. In the native peptide, Trp2 is bromoTrp, and Cys24 is disulfide bonded to a cysteine; i.e., Cys24 is S-cysteinylated (Gajewiak et al. 2014). Two derivatives of the peptide, μO§-GVI11SSG and μO§-GVI11SSC, were synthesized as recently described (Gajewiak et al. 2014) and used in the present study. Trp2 in neither derivative was brominated, and the two derivatives differed from each other in that Cys24 was disulfide bonded either to cysteine (in μO§-GVI11SSG) or glutathione (in μO§-GVI11SSC). (Thus μO§-GVI11SSG differs from the native peptide by only lacking bromination of Trp2.) The two peptides sharing the condensed term, μO§-GVI11SSG, behaved similarly in blocking rNaV1.1s expressed in oocytes (Gajewiak et al. 2014). Their use in a given experiment of the present study was based, in part, on their availability at the time the experiment was performed.

Preparation and voltage clamp of X. laevis oocytes expressing cloned VGSCs. Clones for mNaV1.6 (NM_011323), rNaVβ1 (NM_017288), and rNaVβ2 (NM_012877.1) were obtained from Alan Goldin (University of California, Irvine). The clone for rNaV1.6 (NM_192662.2) was prepared as described previously (Zhang et al. 2013a). Clones for rNaVβ3 (NM_139097.3) and rNaVβ4 (NM_001008880.1) were obtained from Lori Ison (University of Michigan, Ann Arbor). rNaVβ1 and rNaVβ2 DNA were linearized with NotI and transcribed with T7; rNaVβ3 DNA was linearized with XbaI and transcribed with T7; and rNaVβ4 DNA was linearized with BamHI and transcribed with T7.

Oocytes were harvested and prepared essentially as described previously (Cartier et al. 1996). Briefly, freshly excised oocytes were treated with 2.5 mg/ml collagenase A (Roche Diagnostics, Indianapolis, IN) in OR-2 (82.5 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl2, and 5.0 mM HEPES, pH 7.3) for 1–2 h on a rotary shaker at room temperature. Halfway through the collagenase treatment, the solution was exchanged with fresh collagenase solution. Following the enzyme...
treatment, oocytes were rinsed with OR-2 and incubated until used at 16°C in ND96 (96 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, and 5.0 mM HEPES, pH 7.3), supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml).

Injection of cRNA into oocytes was done as described previously (Wilson et al. 2011a; Zhang et al. 2013a). Briefly, a given oocyte was injected with 30–70 nl of 0.3 ng nNaβ, 1.6 or 117 ng rat cRNA in distilled water, or with an equal weight of a nNaβ cRNA. Oocytes were incubated at 16°C for 1–6 days in ND96, supplemented with the aforementioned antibiotics.

Oocytes were two-electrode voltage clamped with an OC-725C amplifier (Warner Instruments, Hamden, CT), using 3 M KCl-filled microelectrodes (<0.5 MΩ resistance), essentially as described previously (Zhang et al. 2013a). A holding potential of ~80 mV was used, and I∞ was induced every 20 s with a 50-ms depolarizing step to −10 mV. Current signals were filtered at 2 kHz, digitized at a sampling frequency of 10 kHz, and leak subtracted with a P/8 protocol using in-house software written in LabVIEW (National Instruments, Austin, TX). The recording chamber was a 4-mm diameter well (total volume of 30 μl), sunk in Sylgard (Dow Corning, Midland, MI), a silicone elastomer. Toxins were dissolved in ND96, and oocytes were exposed to toxin by applying 3 μl toxin solution (at 10X the final concentration) to a static bath with a pipettor and manually stirring the bath for a few seconds by gently aspirating and expelling a few microliters of bath fluid several times with the pipettor. A static bath was used to conserve toxin, and toxins were washed out by continuous perfusion with ND96, initially at a rate of 1.5 ml/min for 20 s and then at a steady rate of 0.5 ml/min.

Oocyte data were analyzed as follows. Percentage block of peak I∞ by toxin was determined by obtaining the average peak of greater than or equal to three control traces and the average peak of greater than or equal to three traces acquired at steady state in the presence of toxin and then dividing the latter by the former and multiplying by 100. Fitting of time-course data to a single exponential function was done with homemade software written with LabVIEW. The interaction of toxin with channel was assumed to be that of a simple bimolecular reaction whose kinetics are described by the equation, koff = k onset [toxin] + koff, where [toxin] is toxin concentration, k onset is the observed association rate constant, and koff is the dissociation rate constant. The time course of peak I∞ was plotted before, during, and after exposure to toxin. The k∞ was determined as follows: the onset of block at a given [toxin] was fit to a single exponential function to yield the k∞, following which k∞ was obtained from the linear-regression slope of a k∞ vs. [toxin] plot for at least three different [toxins] (where each concentration was tested on greater than or equal to three oocytes), as described previously (Zhang et al. 2013a). The k∞ was determined by fitting the toxin-washout curve to a single-exponential function; however, when recovery from block was very slow (<50% recovery after 20 min; i.e., k∞ < 0.035/min), k∞ was estimated from the level of recovery observed after 20 min of washing and assuming recovery followed a single exponential time course. Times longer than 20 min were not used to avoid error due to possible baseline drift. Each k∞ value was the average of greater than or equal to nine oocytes.

**Extracellular recording of CAPs from rat and mouse sciatic nerves.** Preparation of sciatic nerves and recordings from them were performed essentially as described previously (Fiedler et al. 2008; Wilson et al. 2011a). Briefly, sciatic nerves were dissected from adult male Sprague-Dawley rats and Swiss Webster or C57/BL6 mice, desheathed, and used within ~2 h. A given nerve was placed in a multiwell Vaseline-gap chamber, made of Sylgard. Each well was 4 mm in diameter and ~4 mm deep, with ~1 mm wide partitions between adjacent wells. All wells contained mammalian Ringer’s solution consisting of 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1.1 mM MgCl₂, and 5 mM HEPES, pH 7.4. The proximal end of the nerve was in well 1 and the distal end in either well 3 or 4. The portions of the nerve overlying the partitions were covered with Vaseline. The nerve was stimulated with wire electrodes in wells 1 and 2, and CAPs were recorded with a pair of wire electrodes in either wells 2 and 3 or 3 and 4, with the electrode in the lower-numbered well connected to the positive input of the preamplifier. A ground electrode was situated in well 2 or 3. All electrodes were platinum wires. Extracellular records were acquired with a differential capacitive-coupled preamplifier, band-pass filtered (1 Hz–1 kHz) and sampled at 4 kHz using in-house software written in LabVIEW. The amplitude of a 0.1- to 1-ms duration constant-voltage pulse was adjusted to provide a supramaximal stimulus that evoked both A- and C-CAPs, and the stimulus was applied once/minute. Normally, the nerve was exposed to toxin by replacing the solution in a middle well with toxin-containing Ringer’s solution. In experiments with μO§-GVIIJSSC, the Ringer’s solution also contained 0.1 mg/ml BSA to minimize nonspecific binding. [Separate experiments with oocytes showed that the presence of BSA did not affect the peptide’s activity (not illustrated).] In the case of tests with δ-PVIA, which inhibits channel inactivation, the toxin was applied to the last well, i.e., the well with the recording electrode that fed into the negative input of the preamplifier, as described previously (Bulaj et al. 2001). Solutions in all wells were static and manually replaced every 10–15 min with fresh solutions. To minimize evaporation, the atmosphere immediately above the wells was exposed to a gentle stream of water-saturated air.

Following tests of μO§-GVIIJSSC on sciatic nerves, the tested (i.e., “used”) peptide was retrieved from the well and assayed for 1) functional activity on voltage-clamped oocytes expressing nNaβ, 1.6 (see above) and 2) structural integrity by HPLC (see below).

**Whole-cell voltage-clamp recording of I∞ from acutely dissociated DRG neurons from rat.** DRG neurons of adult male Sprague-Dawley rats were dissociated and used as described previously (Zhang et al. 2013b). Briefly, ganglia were excised and treated with collagenase, followed by trypsin. Cells were mechanically dissociated by trituration, washed, and suspended in Leibovitz L-15 medium, supplemented with 14 mM glucose, 1 mM CaCl₂ 10% FBS, and penicillin/streptomycin. Dissociated neurons were kept in suspension at 4°C for up to 3 days (Blair and Bean 2002). Voltage-clamp recordings were performed with a MultiClamp 700A amplifier (Axon Instruments, Union City, CA) using a bath with a total volume of 100 μl, essentially as described previously (Zhang et al. 2013b). The extracellular solution contained 140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.1 mM CdCl₂, and 20 mM HEPES, pH 7.3. Patch pipettes had resistances of ~2 MΩ and contained 140 mM CsF, 10 mM NaCl, 1 mM EGTA, and 10 HEPES, pH 7.3; series resistance compensation was >80%. After the achievement of whole-cell clamp conditions, recordings were not initiated until the holding current had settled, which required >10 min. The contribution of nNaβ, 1.9, relative to that of nNaβ, 1.8, to the TTX-resistant current of DRG neurons is minimized by such a settling period (Choi et al. 2006). The membrane potential was held at ~80 mV, and I∞ were elicited with a 50-ms step to 0 mV, applied every 20 s. Current signals were low-pass filtered at 3 kHz, digitized at a sampling frequency of 10 kHz, and leak subtracted by a P/6 protocol using in-house software written in LabVIEW.

Toxins were dissolved in extracellular solution and applied to the clamped neuron by simple bath exchange that involved manually applying, with a pipette, toxin solution (150 μl) at one end of the boat-shaped, 100-μl chamber, while simultaneously withdrawing solution at the other end of the chamber over a time span of ~20 s. The patch electrode was used to lift the cell from the chamber bottom and position the cell near the upstream half of the chamber to ensure that the cell was fully exposed to the introduced toxin solution. Toxin exposures were conducted in a static bath to conserve toxin. The level of TTX-resistant I∞ of each DRG cell was determined by perfusion with 1 μM TTX following tests with μO§-GVIIJSSC, which does not block nNaβ, 1.8 expressed in oocytes (Gajewski et al. 2014).

Use of animals in this study followed protocols approved by the University of Utah’s Institutional Animal Care and Use Committee.
that conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

All electrophysiological experiments were conducted at room temperature (~22°C).

HPLC of μO$\bar{\Sigma}$-GVII$\bar{\Sigma}$SSG. Qualitative and quantitative analyses of used μO$\bar{\Sigma}$-GVII$\bar{\Sigma}$SSG (see above) were performed with an analytical C$_1$$_8$ Vydc reversed-phase HPLC column (218TP54, 250 mm × 4.6 mm, 5 μm particle size) at 23°C. Solvents consisted of 0.1% (v/v) trifluoroacetic acid in either water (solvent A) or 90% aqueous acetonitrile (solvent B). The sample was eluted with solvent A and a linear gradient of 15–45% solvent B. The optical absorbance of the eluent was monitored at 220 nm.

**RESULTS**

mNaV$_{1.6}$, expressed in oocytes, is blocked by μ-contoxins BuIIIB and SxIIIA more potently than rNaV$_{1.6}$. In previous experiments, we tested μ-BuIIIB and μ-SxIIIA against mNaV$_{1.6}$ expressed in X. laevis oocytes without any NaV$_{\gamma}$-subunit coexpression, and they had similar IC$_{50}$ (1.8 and 0.57 μM, respectively) (Wilson et al. 2011a). All of the experiments described below that involved coexpression with NaV$_{\gamma}$-subunits from rat, because clones from mouse were unavailable to us. Thus we tested μ-BuIIIB and μ-SxIIIA against rNaV$_{1.6}$ and mNaV$_{1.6}$, coexpressed with NaV$_{\gamma}$, and observed that although both peptides had similar affinities for a given channel, each had a higher affinity for mNaV$_{1.6}$ than rNaV$_{1.6}$ (Fig. 1), where the higher affinity of μ-BuIIIB for mNaV$_{1.6}$ is a consequence of a larger $k_{on}$ and smaller $k_{off}$ (Table 1). Values of $k_{on}$ for μ-SxIIIA were too large to be measured with our oocyte system; however, the $k_{off}$ of μ-SxIIIA for the rat channel is similar to that for the mouse channel (Table 1), indicating that the higher affinity of μ-SxIIIA for mNaV$_{1.6}$ results from a larger $k_{on}$ for the mouse, over rat, channel.

The block by μ-SxIIIA of mNaV$_{1.6}$, coexpressed with various combinations of the four NaV$_{\beta}$-isoforms, was examined (Table 2). Coexpression with NaV$_{\beta}$1 or -β3 minimally affected affinity. In contrast, coexpression with NaV$_{\beta}$2 or -β4 increased the IC$_{50}$ >10-fold. This increase in IC$_{50}$ was observed with unary coexpression of NaV$_{\beta}$2 or -β4 was largely “reversed” with binary coexpression of NaV$_{\beta}$2 or -β4 with NaV$_{\beta}$1 or -β3 (Table 2).

μO$\bar{\Sigma}$-GVII$\bar{\Sigma}$SSG and -GVII$\bar{\Sigma}$SSG block mNaV$_{1.6}$, expressed in oocytes, except when NaV$_{\beta}$2 or -β4 is coexpressed. Two synthetic derivatives of the recently discovered μO$\bar{\Sigma}$-GVII

![Graph](https://example.com/graph.png)

**Fig. 1.** Block by μ-BuIIIB and μ-SxIIIA of mouse and rat α-subunit of voltage-gated sodium channels (mNaV$_{1.6}$; mNaV$_{1.6}$ and rNaV$_{1.6}$, respectively), coexpressed with rat β-subunit (rNaV$_{\gamma}$B1) in Xenopus laevis. Oocytes were prepared and voltage clamped as described in MATERIALS AND METHODS. The holding potential was −80 mV, and voltage-gated sodium currents ($I_{\text{Na}}$) were induced by a 50-ms step to −10 mV, applied every 20 s. A–D: each pair of panels shows example time course of block (top; where bars represent when toxin was present) and sample responses (bottom; where control traces are gray and in-toxin traces are black). Block of mNaV$_{1.6}$ by 10 μM μ-BuIIIB (A) or μ-SxIIIA (B) and rNaV$_{1.6}$ by 10 μM μ-BuIIIB (C) or μ-SxIIIA (D). E: percentage block as a function of toxin concentration ([Toxin]) of mNaV$_{1.6}$ (left pair of curves) and rNaV$_{1.6}$ (right pair of curves) with μ-BuIIIB (closed symbols) and μ-SxIIIA (open symbols). Data points are mean ± SD (n = 4 oocytes). Continuous, solid (μ-SxIIIA) and dashed (μ-BuIIIB) curves are fits to the Langmuir adsorption isotherm; IC$_{50}$ values derived from these fits are presented in Table 1.

**Table 1.** Comparison of block by μ-SxIIIA and μ-BuIIIB of rat vs. mouse NaV$_{1.6}$, expressed with rat NaV$_{\gamma}$B1 in Xenopus laevis oocytes*

<table>
<thead>
<tr>
<th>Channel</th>
<th>μ-SxIIIA</th>
<th>μ-BuIIIB</th>
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<tr>
<td></td>
<td>$k_{on}$</td>
<td>μM⁻¹min⁻¹</td>
</tr>
<tr>
<td>rNaV$_{1.6}$ + rβ1</td>
<td>NA$^*$</td>
<td>NA$^*$</td>
</tr>
<tr>
<td>mNaV$_{1.6}$ + rβ1</td>
<td>1.55 ± 0.91</td>
<td>19.89 ± 3.24</td>
</tr>
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*Values are mean ± SD [n = 9 oocytes for association rate constant ($k_{on}$) and IC$_{50}$ values; n = 3 oocytes for dissociation rate constant ($k_{off}$) values], determined as described in MATERIALS AND METHODS. NA, values not available because observed time courses of block at the toxin concentrations tested were too fast to quantify accurately. IC$_{50}$ values from Fig. 1E. rNaV$_{1.6}$, rat α-subunit of voltage-gated sodium channels; NaV$_{\gamma}$B1, β-subunit of voltage-gated sodium channels; rβ1, rat β1; mNaV$_{1.6}$, mouse NaV$_{1.6}$.**
were tested, GVIIJSSC and GVIIJSSG, where the subscripts indicate that cysteine or glutathione, respectively, was disulfide bonded to Cys24 of the peptide (see MATERIALS AND METHODS). mNa1.6 expressed alone was readily blocked by μO§-GVII-JSSC or μO§-GVIIJSSG (Fig. 2, A and J). μO§-GVIIJSSC also readily blocked mNa1.6, coexpressed with either Naβ1 or β3 (Fig. 3, B and D); in contrast, coexpression with Naβ2 or -β4, either alone or in binary combination with Naβ1 or -β3, protected the channels against block by the peptide (Fig. 2, E–J). The kinetic constants of the block by μO§-GVIIJSSC are tabulated in Table 3.

### Table 2. Block by μ-SxIII of mNa1.6 expressed alone or coexpressed with various combinations of the 4 isoforms of rNaαβ-subunits

| mNa1.6 | k_off min−1 | IC50 μM* | % Block by 10 (or 30) μM | μSxIIIαβ
<table>
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<tbody>
<tr>
<td>Alone</td>
<td>0.18 ± 0.06f</td>
<td>0.57 ± 0.08f</td>
<td>95 (98)</td>
<td></td>
</tr>
<tr>
<td>+β1</td>
<td>1.10 ± 0.14f</td>
<td>0.48 ± 0.04f</td>
<td>95 (98)</td>
<td></td>
</tr>
<tr>
<td>+β2</td>
<td>4.92 ± 0.72</td>
<td>7.74 ± 0.12</td>
<td>56 (80)</td>
<td></td>
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<tr>
<td>+β3</td>
<td>4.80 ± 1.30</td>
<td>0.44 ± 0.01</td>
<td>96 (99)</td>
<td></td>
</tr>
<tr>
<td>+β4</td>
<td>3.80 ± 1.21</td>
<td>11.21 ± 0.30</td>
<td>47 (73)</td>
<td></td>
</tr>
<tr>
<td>+β1 +β2</td>
<td>3.53 ± 1.40</td>
<td>2.32 ± 0.03</td>
<td>82 (93)</td>
<td></td>
</tr>
<tr>
<td>+β1 +β4</td>
<td>2.47 ± 0.35</td>
<td>1.05 ± 0.05</td>
<td>91 (97)</td>
<td></td>
</tr>
<tr>
<td>+β3 +β2</td>
<td>4.21 ± 1.59</td>
<td>1.15 ± 0.01</td>
<td>90 (96)</td>
<td></td>
</tr>
<tr>
<td>+β3 +β4</td>
<td>4.74 ± 0.80</td>
<td>1.08 ± 0.02</td>
<td>90 (97)</td>
<td></td>
</tr>
</tbody>
</table>

*Values are mean ± SD (n = 9 oocytes for IC50 values; n = 3 oocytes for k_off values). Values of k_off are not available because the observed association rate constant at the concentrations tested was too large to measure. Predicted percentage of channels blocked from the Langmuir equation, % block = 100%/(1 + CIC50), where C = 10 μM (or 30 μM for values in parentheses). fFrom Wilson et al. (2011a). From Table 1.

TTX (0.1 μM) blocked 100% of the A-CAPs and ~80% of the C-CAPs (Table 4), where the conduction velocity of the highly attenuated C-CAPs was decreased markedly (Fig. 3H). The residual amplitude of C-CAPs that persisted presumably reflects the presence of Naα1.8 and/or -1.9, which are TTX resistant, in C-fibers. These TTX results essentially mirror those observed with A- and C-CAPs of the rat sciatic nerve (Wilson et al. 2011a).

A- and C-CAPs in mouse and rat sciatic nerves are not blocked by μO§-GVIIJSSC or -GVIIJSSG. Neither μO§-GVII-JSSC nor μO§-GVIIJSSG was able to block A- and C-CAPs in mouse and rat sciatic nerves at 33 μM (Fig. 4, A and B), a concentration well beyond that necessary to block the majority of the INa of mNa1.6, provided it wasn’t coexpressed with Naβ2 or -β4 (Fig. 2 and Table 3). In addition, inspection of Fig. 4 also shows that μO§-GVIIJSSC/G produced no increase in the latency of A- or C-CAP, indicating no decrease in the conduction velocity of action potentials in A- or C-fibers.

To examine whether the peptide was degraded or absorbed by the tissue to which it was exposed, the used toxin solution was retrieved and examined for structural integrity by subjecting it to HPLC analysis (Fig. 4C), as well as for functional activity against mNa1.6 expressed in oocytes, which was performed as follows. A sample of used GVIIJSSC solution was diluted 10-fold with ND96 and assayed on an oocyte expressing mNa1.6. The k_off of INa was 3.0 min−1, which (after accounting for the 10-fold dilution) is that expected of a GVIIJSSC concentration of 26 μM, a value 21% lower than the starting 33 μM. The slightly (20–30%) lower-than-expected values obtained with the HPLC and oocyte assays can be explained by dilution due to siphoning of Ringer’s solution from adjacent wells during retrieval of the contents from the toxin-containing well. Thus by both structural and functional assays, the used toxin proved to be intact and largely recoverable.

δ-PVIA affects A-CAPs of the mammalian sciatic nerve, like members of all other families of conotoxins that target VGSCs except μO§-conotoxins. As mentioned previously, there are five families of conotoxins that target VGSCs. Until now, all families, except μO§- and δ-conotoxins, have been shown to affect A-CAPs of the mammalian sciatic nerve (see DISCUSSION). Earlier experiments with δ-PVIA and -SVIE showed that these peptides prolonged the action potentials in peripheral nerves of frogs (Rana pipiens) (Bulaj et al. 2001; West et al. 2005), and here, we examined whether δ-PVIA could be shown also to act on the mammalian (specifically, rat) sciatic nerve. Indeed, 10 μM δ-PVIA greatly prolonged the A-CAP (Fig. 5). The prolonged A-CAPs persisted longer than the latency of the C-CAPs and therefore, rendered accurate characterization of the latter problematical, so the effects of δ-PVIA on C-CAPs remain to be determined. The effect of δ-PVIA on A-CAPs was very robust and distinctive and closely resembled that seen with δ-SVIE on A-CAPs of frog nerve [see Fig. 3B in Bulaj et al. (2001)]. The profound potentiation and prolongation of the upward phase of A-CAPs induced by δ-PVIA, illustrated in Fig. 5, were observed in duplicate (two out of two) trials but never in traces of countless trials without the peptide [e.g., see traces in Fig. 4, as well as Fig. 3, of Wilson et al. (2011a)]. This result supports the notion that peptide inaccessibility is an unlikely cause for the lack of activity of μO§-GVIIJSSC on the sciatic nerve, as recounted in DISCUSSION.
Fig. 2. μO§-GVII\textsubscript{SSG} (where subscript refers to cysteinylated) readily blocks mNa\textsubscript{v1.6}, expressed alone or coexpressed with rNa\textsubscript{v}β1 or β3; however, coexpression with rNa\textsubscript{v}β2 or β4 protects mNa\textsubscript{v1.6} against block by μO§-GVII\textsubscript{SSG}. Oocytes were voltage clamped, as described in Fig. 1. A–J: there are 10 pairs of panels. Top: representative time course of block before, during, and after exposure to GVII\textsubscript{SSG} (A–F) or GVII\textsubscript{SSC} (where subscript refers to glutathionylated; J), where the bar represents when peptide was present (thin black bars, 1 M; thick black bars, 33 μM). Bottom: sample responses before (light traces) and during (dark traces) toxin application. First and last pair of panels represent mNa\textsubscript{v1.6} expressed alone (A and J), and the remaining 8 pairs of panels are labeled with the rNa\textsubscript{v}β-subunits that were coexpressed with mNa\textsubscript{v1.6} (B–I). GVII\textsubscript{SSG} (1 μM) readily blocked mNa\textsubscript{v1.6} expressed alone (A) or coexpressed with either Na\textsubscript{v}β1 (B) or Na\textsubscript{v}β3 (D); in contrast, 33 μM GVII failed to block whenever Na\textsubscript{v}β2 or β4 was coexpressed in either unary fashion (C and E) or binary combination with Na\textsubscript{v}β1 (F and G) or β3 (H and I). (Kinetic constants derived from replicate experiments are presented in Table 3). J: block of mNa\textsubscript{v1.6} (no Na\textsubscript{v}β-subunit coexpressed) by 10 μM GVII\textsubscript{SSG}; time course (top; bar represents when peptide was present) and representative traces (bottom).

μO§-GVII\textsubscript{SSG} blocks I\textsubscript{Na} of small, but not large, dissociated rat DRG neurons. The inability of μO§-GVII\textsubscript{SSC} to block CAPs raises the question of whether these peptides had any activity on endogenous VGSCs. To examine this issue, three small and three large dissociated rat DRG neurons were whole-cell patch clamped as before (Zhang et al. 2013b) and subjected to 10 μM μO§-GVII\textsubscript{SSG}. The peptide blocked, albeit partially, TTX-sensitive I\textsubscript{Na} of all three small neurons; in contrast, all three large neurons were insensitive to the peptide (Fig. 6). Similar tests with μO§-GVII\textsubscript{SSG} remain to be performed.

DISCUSSION

In this report, we examined the effects primarily of two conotoxin families, μ- and μO§-conotoxins, on VGSCs in...
three preparations: 1) *X. laevis* oocytes exogenously expressing Na\(_{\alpha}v\).1.6, with or without various Na\(_{\alpha}v\).\(\beta\)-isoforms; 2) A- and C-fibers of rodent sciatic nerves; and 3) soma of dissociated small and large neurons of rat DRG.

\(\mu\)-Conotoxin sensitivities of rNa\(_{\alpha}v\).1.6 vs. mNa\(_{\alpha}v\).1.6. \(\mu\)-BuIIIB and \(\mu\)-SxIIIA blocked Na\(_{\alpha}v\).1.6 of rat and mouse with IC\(_{50}\) values that differ by >50-fold (Fig. 1E and Table 1). This is in contrast to two other \(\mu\)-conotoxins for which affinity data for both rat and mouse are available; namely, \(\mu\)-SmIIIA and \(\mu\)-PIIIA, each of whose dissociation constants for rNa\(_{\alpha}v\).1.6 vs. mNa\(_{\alpha}v\).1.6 differ by a factor of only 2.3 or less (Wilson et al. 2011a; Zhang et al. 2013a). rNa\(_{\alpha}v\).1.6 and mNa\(_{\alpha}v\).1.6 differ in 10
residues, six of which are in the extracellular portions of the channel. Of these, three are in the pore loop regions: one in DII and two in DIII. The residue in question in DII is located near the N-terminal end of the S5–S6 (pore) loop: Asn907 in rNaV1.4 and Ser907 in mNaV1.6. The homologous residue in rNaV1.4 is Ala728, and the activity of μ-conopeptide GIIGA, a potent blocker of rNaV1.4 (Cruz et al. 1985), has been tested extensively against mutants of rNaV1.4 by several investigators, and a subset of those studies shows that mutations of Ala728 do influence the binding of μ-GIIIA and its congener, μ-GIIIB (Chahine et al. 1998; Li et al. 2003). Furthermore, it was recently shown that the block of rNaV1.4 by μ-SIIIA is reduced significantly by an A728N mutation (Leipold et al. 2014), all of which happen to be sensitive to TTX. It should be noted that the results from coexpression of an NaV1.1-isoform and that from mNaV1.6—there is a two-residue difference for β1, one for β3, six for β2, and five for β4—so the results from coexpression of an NaV1.1-isoform from one rodent species with an NaV1.6-isoform from another species should be viewed with caution.

**Table 3. Block by μO§-GVIIJ SSC of mNaV1.6 expressed alone or coexpressed with various combinations of the 4 isoforms of rNaV1-β-subunits**

<table>
<thead>
<tr>
<th>mNaV1.6</th>
<th>k_{app}, μM⁻¹min⁻¹</th>
<th>k_{off}, min⁻¹</th>
<th>K_{app}, μM⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alone</td>
<td>1.15 ± 0.08</td>
<td>0.069 ± 0.026</td>
<td>0.060 ± 0.023</td>
</tr>
<tr>
<td>+β1</td>
<td>1.42 ± 0.06</td>
<td>0.042 ± 0.023</td>
<td>0.029 ± 0.016</td>
</tr>
<tr>
<td>+β2</td>
<td>NA‡</td>
<td>NA‡</td>
<td>NA‡</td>
</tr>
<tr>
<td>+β3</td>
<td>1.69 ± 0.08</td>
<td>0.093 ± 0.025</td>
<td>0.055 ± 0.015</td>
</tr>
<tr>
<td>+β4</td>
<td>NA‡</td>
<td>NA‡</td>
<td>NA‡</td>
</tr>
<tr>
<td>+β1 + β2</td>
<td>NA‡</td>
<td>NA‡</td>
<td>NA‡</td>
</tr>
<tr>
<td>+β1 + β4</td>
<td>NA‡</td>
<td>NA‡</td>
<td>NA‡</td>
</tr>
<tr>
<td>+β3 + β2</td>
<td>NA‡</td>
<td>NA‡</td>
<td>NA‡</td>
</tr>
<tr>
<td>+β3 + β4</td>
<td>NA‡</td>
<td>NA‡</td>
<td>NA‡</td>
</tr>
</tbody>
</table>

*Values are mean ± SD (n = 9 oocytes for k_{app} values; n = 3 oocytes for k_{off} values). K_{app} dissociation constant; determined from k_{off}/k_{app} * Block percentage value (mean ± SD) compared with average of last 4 traces in the presence of peptide compared with average of 5 traces just before exposure to peptide. t_{1/2}, time to block by 33.3%. Significantly different from no block: ⋆P < 0.05, ⋆P < 0.001 (1-tail Student’s t-test, assuming % block cannot be <0). §Expected block percentage of mNaV1.6 with no NaV1-β-subunit coexpressed in X. laevis oocytes from Supplemental Table 5 of Wilson et al. (2011a). A-CAPs, large, myelinated A-fiber compound action potentials; C-CAPs, small, unmyelinated C-fiber CAPs.

Despite the congruity of results described immediately above, it should be noted that there are differences in the sequences of a given rNaV1-β-isoform and that from mNaV1.6—there is a two-residue difference for β1, one for β3, six for β2, and five for β4—so the results from coexpression of an NaV1.1-isoform from one rodent species with an NaV1.6-isoform from another species should be viewed with caution.

**Modulation of the sensitivity of mNaV1.6 to μO§-GVIIJ SSC by coexpression of rNaV1-β-subunits.** The recently discovered μO§-GVIIJ is the charter member of a new, fifth family of conotoxins that target VGSCs. Until now, it has been tested only on rat VGSCs expressed in oocytes, where it readily blocks rNaV1.1, -1.2, -1.3, -1.4, -1.6, and -1.7 (Gajewiak et al. 2014), all of which happen to be sensitive to TTX. It should be noted that the TTX sensitivity is largely dictated by an aromatic residue in site 1 near the ion-selectivity filter (Santarelli et al. 2007), whereas the μO§-GVIIJ sensitivity is dictated by a Cys residue in the newly described site β between S5 and SSS (the latter is the proximal limb of the pore loop) of DII (Gajewiak et al. 2014).

By and large, μO§-GVIIJ SSC and μO§-GVIIJ SSG behave similarly, both in previous (Gajewiak et al. 2014) and present experiments. A subtle difference is that the on rate of μO§-GVIIJ SSC is apparently larger than that of μO§-GVIIJ SSG, which is evident in Fig. 2 (compare pairs in Fig. 2A with Fig. 2J, where in mind the difference in peptide concentrations). This is also the case with rNaV1.2 and -1.7, where the k_{on} of μO§-GVIIJ SSC is approximately threefold larger than that of μO§-GVIIJ SSG (Gajewiak et al. 2014). Cysteine is smaller than glutathione, and this may explain the difference in k_{on}. A detailed examination of this issue is under way with derivatives of μO§-GVIIJ, where the moiety disulfide bonded to Cys24 of

**Table 4. Kinetics and levels of block of A- and C-CAPs of mouse sciatic nerve by 7 μ-conopeptides; comparison with expected block of mNaV1.6 expressed in X. laevis oocytes**

<table>
<thead>
<tr>
<th>Toxin (μM)</th>
<th>A-CAPs</th>
<th>C-CAPs</th>
<th>mNaV1.6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Block*</td>
<td>t_{1/2}, min⁻¹</td>
<td>% Block*</td>
</tr>
<tr>
<td>μ-SmIIIA (10)</td>
<td>100 ± 0§</td>
<td>2.2</td>
<td>62.6 ± 2.2§</td>
</tr>
<tr>
<td>μ-KIIIA (10)</td>
<td>100 ± 0§</td>
<td>2.8</td>
<td>38.7 ± 4.3§</td>
</tr>
<tr>
<td>μ-SIIIA (10)</td>
<td>100 ± 0§</td>
<td>15</td>
<td>41.6 ± 2.1§</td>
</tr>
<tr>
<td>μ-GIIIA (10)</td>
<td>100 ± 0§</td>
<td>1.1</td>
<td>0.8 ± 2.2 N/A</td>
</tr>
<tr>
<td>μ-PIIIA (10)</td>
<td>100 ± 0§</td>
<td>1 &lt;</td>
<td>2.6 ± 4.7 N/A</td>
</tr>
<tr>
<td>μ-SIIIa (10)</td>
<td>69 ± 3 §</td>
<td>&lt;1</td>
<td>0.8 ± 4.24 N/A</td>
</tr>
<tr>
<td>μ-Bullib (10)</td>
<td>94.1 ± 4.§</td>
<td>6</td>
<td>17.5 ± 8.0§</td>
</tr>
<tr>
<td>TTX (0.1)</td>
<td>100 ± 0§</td>
<td>&lt;1</td>
<td>82.4 ± 4.7§</td>
</tr>
</tbody>
</table>
the peptide varies over a range of sizes and charges (unpublished observations).

A stellar feature of μO§-GVIIJ_{SSC/G} is that coexpression with Na\textsubscript{v}β2 or -β4 renders otherwise-susceptible rNa\textsubscript{v}1.6 resistant to the peptide (Gajewiak et al. 2014). We now show that this modulation by coexpression of Na\textsubscript{v}β2 or -β4 also applies to mNa\textsubscript{v}1.6 (Fig. 2 and Table 3). Furthermore, the protection of mNa\textsubscript{v}1.6 against μO§-GVIIJ\textsubscript{SSG} block by coexpression with Na\textsubscript{v}β2 or -β4 persists in the face of binary coexpression with Na\textsubscript{v}β1 or -β3; i.e., the effects of Na\textsubscript{v}β2 or -β4 coexpression dominate those of Na\textsubscript{v}β1 or -β3 (Table 3). Similarly, dominance of the coexpression of Na\textsubscript{v}β2 and -β4 over that of Na\textsubscript{v}β1 was observed with rNa\textsubscript{v}1.2 (Gajewiak et al. 2014). This is unlike the effects of Na\textsubscript{v}β2 or -β4 coexpression on the block of mNa\textsubscript{v}1.6 by μ-SxIIIA, which are attenuated by binary coexpression with Na\textsubscript{v}β1 or -β3 (Table 2). As mentioned in INTRODUCTION, Na\textsubscript{v}β2 and -β4 are disulfide bonded to the α-subunit. The binding site of μO§-GVIIJ on Na\textsubscript{v}1, site 8, has a Cys residue with which we hypothesized the peptide can form a disulfide bond; furthermore, we speculated that Na\textsubscript{v}β2 and -β4 may protect the channel against block by being disulfide bonded with the Cys at site 8 (Gajewiak et al. 2014). Further investigation of this possibility is underway.

It should be emphasized that the modulatory effect of coexpression of α- with β-subunits does not necessarily mean that the modulation results from the subunits’ physical association per se; for example, Na\textsubscript{v}β1 coexpression can affect the glycosylation of the α-subunit (Laedermann et al. 2013). Thus in principle, the alteration in the pharmacology of a VGSC could result from the coexpression of the β-subunit altering the processing of the channel.

Fig. 4. A- and C-CAPs of rat and mouse sciatic nerves were not blocked by 33 μM μO§-GVIIJ\textsubscript{SSC} or -GVIIJ\textsubscript{SSG}. CAPs were evoked, recorded, and illustrated, essentially as in Fig. 3. A and B: top 2 plots in each 4-plot panel show time course of normalized peak-to-peak amplitudes of simultaneously recorded A-CAPs (left) and C-CAPs (right) from rat (A) and mouse (B) sciatic nerves, where the bars above each plot indicate when 33 μM μO§-GVIIJ\textsubscript{SSC} was present (note absence of data point near minute 5, when μO§-GVIIJ\textsubscript{SSC} was being applied), and arrows depict when 10 μM μ-SmlIIA was introduced (note compressed time base for data points representing responses in μ-SmlIIA). Data points for μO§-GVIIJ\textsubscript{SSC} represent mean ± SE values (n = 3 sciatic nerves), and those for μ-SmlIIA are from single nerves (i.e., n = 1). Bottom 2 plots in each 4-plot panel show example traces of A- and C-CAPs obtained in control solution (dashed traces), in 33 μM μO§-GVIIJ\textsubscript{SSC} (solid traces, essentially same as control traces), and in 10 μM SmlIIA (dotted traces, where A-CAP was totally blocked, while C-CAP was dramatically slowed and attenuated). The shaded area at the start of the mouse A-CAP trace contains the stimulus artifact and can be ignored. Toward the end of a rat nerve trial, 33 μM GVIIJ\textsubscript{SSC} was also applied, and still, no block of A- and C-CAPs was seen (not illustrated). C: the “used” GVIIJ\textsubscript{SSC} solution from a rat trial was retrieved at the end of the experiment and subjected to analysis by HPLC (see MATERIALS AND METHODS). The elution profile consisted of a single peak with the same retention time as unused GVIIJ\textsubscript{SSC} (Gajewiak et al. 2014), showing that the peptide remained intact, and integration under the peak indicated that ~0.8 nmol was applied to the column, a value 29% lower than the expected 1.1 nmol. A\textsubscript{220}, absorbance at 220 nm.
Effects of $\mu$-BuIIIB, $\mu$-SxIII/A, and five other $\mu$-conotoxins on A- and C-CAPs of mouse sciatic nerve are consistent with NaV1.6 and -1.7 mediating action potentials in A- and C-fibers, respectively. In previous experiments, we used a panel of $\mu$-conotoxins and concluded that NaV1.6 and -1.7 were the major NaV1.1-isomers responsible for the conduction of action potentials in A- and C-fibers, respectively, of rat sciatic nerve (Wilson et al. 2011a). Results in Fig. 3 and Table 4 lead us to conclude the same for the mouse sciatic nerve. We do not have a clone of mNaV1.7, so we assume that mNaV1.7 behaves similar to rNaV1.7 toward the tested $\mu$-conotoxins, with the possible exception of $\mu$-SII/A, which we speculated in RESULTS may block rNaV1.7 better than mNaV1.7. This issue can be resolved when an mNaV1.7 clone becomes available.

The safety factor for action potential conduction can be defined as the current generated during an action potential divided by the threshold current necessary for the action potential to be propagated (Fern and Harrison 1993) or for our purposes, the density of sodium channels available for activation during an action potential divided by the minimum density of channels necessary for action potential conduction. In view of the safety factor, normally, a disproportionate fraction of sodium channels must be blocked before the action potential is blocked. For example, the IC$_{50}$ values of $\mu$-SxIII/A in blocking rNaV1.6+$\beta$1 and mNaV1.6+$\beta$1 are 20 and 0.5 $\mu$M, respectively (Table 1). Given these IC$_{50}$ values, the Langmuir equation (see Table 2) predicts that 10 $\mu$M SxIII/A would block 95% and 33% of the mouse and rat channels, respectively. On the other hand, our experiments show that 10 $\mu$M SxIII/A blocked $\sim$70% of the A-CAPs in mouse (Table 4) and essentially 0% of those in rat [see Table 3 of Wilson et al. (2011a)]. We do not know the safety factor for the conduction of A-CAPs in our experiments; however, the predicted block of NaV1.6-containing VGSCs vs. the observed block of A-CAPs by 10 $\mu$M SxIII/A can be qualitatively reconciled if we assume that the safety factor is approximately two for both mouse and rat nerves; that is, $>50\%$ of the channels must be blocked for A-CAPs to fail. The disparity in the block of channels vs. action potentials would be larger, the larger the safety factor. This analysis requires several assumptions and does not rule out the possible contribution of minor NaV1.1-isomers to A-CAPs.

Inability of $\mu$O§-GVIIJ$_{SSC/G}$ to block A- and C-CAPs in rat and mouse sciatic nerves suggests that channels expressed in A- and C-fibers are associated with NaV1.2 or -1.4. GVIIJ$_{SSC}$ and GVIIJ$_{SSG}$ blocked neither A- nor C-CAPs in rat and mouse sciatic nerves (Fig. 4). In principle, there are four relatively simple alternative reasons why no block was observed with $\mu$O§-GVIIJ$_{SSC/G}$; namely, the peptide is 1) physically unable to reach to the axon surface where VGSCs reside; 2) absorbed by the tissue preparation; 3) rapidly degraded; or 4) unable to block endogenously expressed VGSCs. To address the first possibility, at the end of a trial with each of a mouse and rat sciatic nerve, the nerve was exposed to 10 $\mu$M $\mu$-SmIII/A, which readily blocked both A- and C-CAPs (Fig. 4), as was invariably observed in multiple experiments with sciatic nerves of both mouse (Fig. 3A and Table 4) and rat (Wilson et al. 2011a). In addition to $\mu$-SmIII/A, many other $\mu$-conotoxins are able to block A- and/or C-CAPs in sciatic nerves of mouse (Fig. 3 and Table 4) and rat (Wilson et al. 2011a). Furthermore, aside from $\mu$O§-GVIIJ$_{SSC/G}$, action potentials in A- and/or C-fibers of the mammalian sciatic nerve are susceptible to members of all other families of conotoxins that target VGSCs, i.e., $\mu$O-conopeptides (Bulaj et al. 2006), $\nu$-conopeptides (Fiedler et al. 2008), and $\delta$-conopeptides (Fig. 5). It should be noted that $\mu$O$-\delta$-conopeptides are more hydrophobic than $\mu$O$-\nu$-conotoxins, whereas $\nu$-conotoxins are larger than $\mu$O$-\nu$-conotoxin. Thus our overall results suggest that a peptide-accessibility issue is unlikely with $\mu$O§-GVIIJ$_{SSC/G}$.

To address the second and third possibilities, after one of the trials in Fig. 3, the used $\mu$O§-GVIIJ$_{SSC}$, was recovered and observed to be structurally intact (Fig. 4C) and functionally active (see RESULTS regarding Fig. 4), which shows that $\mu$O§-GVIIJ$_{SSC}$ was not appreciably absorbed or degraded during exposure to tissue.

To address the fourth possibility, we examined dissociated rat DRG neurons. $\mu$O§-GVIIJ$_{SSG}$ partially blocked the I$_{Na}$ of small neurons, whereas the I$_{Na}$ of large neurons were spared (Fig. 6). This showed that at least some endogenous channels are susceptible to the peptide. A- and C-CAPs arise from fast- and slow-conducting axons of neurons with, respectively, large and small cell somas in DRG (Harper and Lawson 1985); thus the resistance of the I$_{Na}$ of large cells to $\mu$O§-GVIIJ$_{SSG}$ (Fig. 6) is consistent with the resistance of A-CAPs to the peptide.
DRG neurons expressing Na\textsubscript{\(\alpha_1\)6} (Cummings et al. 2005). Thus our results would predict that channels exhibiting resurgent currents would be resistant to \(\mu\text{-SVIIJSSC/G}\); conversely, channels that do not exhibit resurgent currents and yet are resistant to \(\mu\text{-SVIIJSSC/G}\) may be associated with Na\textsubscript{\(\beta_2\)}.

However, this assessment may be too simplistic in light of a recent report that a critical regulator of a resurgent current in cerebellar Purkinje neurons is intracellular FGF14 (Yan et al. 2014).

The block by saturating concentrations of \(\mu\text{-SVIIJSSC/G}\) of various Na\textsubscript{\(\alpha_1\)s} expressed in oocytes is not 100%; i.e., there remains a "residual current" (Gajewiak et al. 2014). It is presently unknown how much of the incomplete block of the TTX-sensitive \(I_{\text{Na}}\) of small neurons by \(\mu\text{-SVIIJSSC/G}\) (Fig. 6) is due to channel heterogeneity and how much to partial efficacy. Heterogeneity in the oxidation state of the Cys residues of the channel can contribute to incomplete block, insofar as DTT treatment of Na\textsubscript{\(\alpha_1\)s} expressed in oocytes reduces the residual current (Gajewiak et al. 2014); thus it would be interesting to see whether DTT treatment of DRG neurons or sciatic nerve can likewise increase the efficacy of block of endogenous channels by \(\mu\text{-SVIIJSSC/G}\). In this regard, it might be noted that we have not determined the safety factor for the propagation of A- and C-CAPs under our experimental conditions; however, as mentioned in RESULTS in describing Fig. 4, \(\mu\text{-SVIIJSSC/G}\) produced no decrease in action potential conduction velocity; in other words, no evidence for partial block was detected.

Thus our current working hypothesis is that coexpression of Na\textsubscript{\(\alpha_1\)6} and/or Na\textsubscript{\(\beta_4\)} protects Na\textsubscript{\(\alpha_1\)s} in both A- and C-fibers against block by \(\mu\text{-SVIIJ}\). This proposition is concordant with other observations; that is, immunohistochemistry, PCR, and in situ hybridization indicate that all four Na\textsubscript{\(\alpha_1\)s} are expressed in DRG neurons (Coward et al. 2001; Takahashi et al. 2003; Yu et al. 2003) with Na\textsubscript{\(\alpha_1\)6} in A-fibers and Na\textsubscript{\(\alpha_1\)s} in both large and small DRG neurons (Ho et al. 2012). Furthermore, the majority of the nodes of Ranvier in rat peripheral nerve is labeled by antibody against Na\textsubscript{\(\alpha_1\)6} (Buffington and Rasband 2013), and in view of our results, the nodes not expressing Na\textsubscript{\(\alpha_1\)6} presumably have VGSCs associated with Na\textsubscript{\(\beta_2\)} instead. If we accept that Na\textsubscript{\(\alpha_1\)6} and/or Na\textsubscript{\(\beta_4\)} is associated with Na\textsubscript{\(\alpha_1\)6} in A-fibers, then it seems reasonable to suggest that Na\textsubscript{\(\alpha_1\)b} or -\(\beta_3\) is also part of the VGSC complex in view of the influence that binary coexpression of Na\textsubscript{\(\alpha_1\)s} and Na\textsubscript{\(\beta_3\)} has on percentage block of mNa\textsubscript{\(\alpha_1\)6} by \(\mu\text{-SxIII}\). Block by 30 \(\mu\text{M} \mu\text{-SxIII}\) of mNa\textsubscript{\(\alpha_1\)6} coexpressed with \(\beta_4\) is expected to be only 73%, whereas 97% block is expected if the channel were coexpressed with \(\beta_1+\beta_4\) or \(\beta_3+\beta_4\) (Table 2). \(\mu\text{-SxIII}\), at concentrations of 30 \(\mu\text{M}\), blocked mouse A-CAPs by \(\sim 95\%\) (Fig. 3I). In view of the safety factor for conduction of action potentials, a disproportionate fraction of sodium channels must be blocked relative to the reduction of the CAP; thus for \(\mu\text{-SxIII}\) to be as potent as it is in blocking A-CAPs in mouse sciatic, Na\textsubscript{\(\alpha_1\)6} in A-fibers is likely to be associated with \(\beta_1\) or \(\beta_3\) in addition to \(\beta_2\) or \(\beta_4\).

We assume here that the \(\mu\text{-conotoxin}\) susceptibilities of VGSCs exogenously expressed in oocytes can be applied directly to channels endogenously expressed in neurons and axons, as we have done previously (Wilson et al. 2011a; Zhang et al. 2013b). \(\mu\text{-Conotoxins}\) bind to site 1 near the selectivity

\textbf{Fig. 6.} \(I_{\text{Na}}\) of small, but not large, dorsal root ganglia neurons are blocked by 10 \(\mu\text{M} \mu\text{-SVIIJSSC/G}\). Acutely dissociated neurons were whole-cell patch clamped, as described in MATERIALS AND METHODS. The holding potential was \(-80\) mV, and \(I_{\text{Na}}\) was induced by a 50 ms step to 0 mV, applied every 20 s. A: percentage block by SVIIJSSC/G of TTX-sensitive \(I_{\text{Na}}\) of 3 small and 3 large neurons; neuron sizes were determined quantitatively by membrane capacitance (see x-axis) and qualitatively by visual inspection. B: 4-plot panel showing representative traces of small (top left) and large (top right) neurons before (light traces) and during (dark traces) exposure to SVIIJSSC/G. Peak \(I_{\text{Na}}\) of these neurons plotted as a function of time (bottom); bars represent when neuron was exposed to SVIIJSSC/G. Illustrated are results of the 12.6-pF small neuron (bottom left) and 45.8-pF large neuron (bottom right). The \(I_{\text{Na}}\) of all neurons was blocked essentially completely by 0.1 \(\mu\text{M} \text{TTX}\), except for the 11.1-pF neuron, which was blocked by 60% (not illustrated).
filter and block the channel by interfering with Na\(^+\) conduction (Cestèle and Catterall 2000; Zhang et al. 2009, 2010a, b). On the other hand, \(\mu\)-O-GVII\textsubscript{SSC}SSG binds to site 8 between S5 and S5S of the pore loop of DII, although its mechanism of block remains to be established (Gajewiak et al. 2014). Thus \(\mu\)- and \(\mu\)-O\(\gamma\)-conotoxins (both of which are relatively hydrophilic) bind to extracellular aspects of the channel and may therefore be minimally influenced by the lipid composition of surrounding the plasma membrane. In this regard, it might be noted that the Na\(_{\alpha,1}\)-isoform selectivity of \(\mu\)-O-GVII\textsubscript{SG} is similar whether the channels are expressed in \textit{X. laevis} oocytes or mammalian cell lines (human embryonic kidney 293 and Chinese hamster ovary cells) (Gajewiak et al. 2014).

Although it is most parsimonious to assume that coexpression of Na\(_{\alpha}\)\(_{\beta}\)2 or \(\beta\)4 is responsible for protecting the endogenous VGSCs from block by \(\mu\)-O-GVII\textsubscript{SSC}G, we cannot rule out that some other factor confers resistance of A- and C-CAPs to \(\mu\)-O\(\gamma\)-GVII\textsubscript{SSC}G; e.g., the efficacy of the peptide in blocking endogenously expressed VGSCs that are not coexpressed with Na\(_{\alpha}\)\(_{\beta}\)2 or \(\beta\)4 may be sufficiently small and/or the safety factor for propagation of action potentials sufficiently large that the inhibition or slowing down of action potentials by \(\mu\)-O\(\gamma\)-GVII\textsubscript{SSC}G is not observable in CAPs. More sensitive means to monitor the effects of toxins on action potentials in the sciatic nerve are under investigation, such as experiments under reduced concentrations of extracellular Na\(^+\) or in the presence of low concentrations of TTX (Colquhoun and Ritchie 1972; Fern and Harrison 1993), as well as experiments to see whether \(\mu\)-O\(\gamma\)-GVII\textsubscript{SSC}G alters the stimulus strength required to generate action potentials following placement of the toxin in the well containing the depolarizing stimulus electrode.

Thus far, we have examined only VGSCs on the cell bodies and bulk of the axons in the peripheral nervous system, and it would be interesting to examine whether \(\mu\)- and \(\mu\)-O\(\gamma\)-conotoxins can be used to assess the likely molecular composition of VGSCs at nerve terminals, as well as at various locations of central nervous system neurons.

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AUTHOR CONTRIBUTIONS

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α- AND β-SUBUNIT OF Na CHANNELS IN RODENT SCIATIC NERVE


