Covalent linkage of bacterial voltage-gated sodium channels

Authors: Huaping Sun[#], Zeyu Zheng[#], Olena A. Fedorenko[¶], Stephen K. Roberts*

* corresponding author; [#] equal contribution

Address: Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster, LA1 4YQ, UK. [¶] present affiliation: School of Life Sciences, University of Nottingham, Nottingham, NG7 2UH, UK.

Author affiliations and emails:

Huaping Sun: huapingsun666@gmail.com; No current affiliation. UK

Zeyu Zheng: Zeyu.zheng@outlook.com; No current affiliation. China

Olena Fedorenko: olena.fedorenko@nottingham.ac.uk; School of Life Sciences, University of Nottingham, Nottingham, NG7 2UH, UK

Address for correspondence (Stephen K Roberts):

Division of Biomedical and Life Sciences,

Faculty of Health and Medicine,

Lancaster University,

Lancaster, LA1 4YQ,

UK.

Tel: 01524 593145

Fax: 01524 843854

Email: s.k.roberts@lancaster.ac.uk

Abstract

Background: Bacterial sodium channels are important models for understanding ion permeation and selectivity. However, their homotetrameric structure limits their use as models for understanding the more complex eukaryotic voltage-gated sodium channels (which have a pseudo-heterotetrameric structure formed from an oligomer composed of four domains). To bridge this gap we attempted to synthesise oligomers made from four covalently linked bacterial sodium channel monomers and thus resembling their eukaryotic counterparts.

Results: Western blot analyses revealed NaChBac oligomers to be inherently unstable whereas intact expression of NavMs oligomers was possible. Immunodectection using confocal microscopy and electrophysiological characterisation of NavMs tetramers confirmed plasma membrane localisation and equivalent functionality with wild type NavMs channels when expressed in human embryonic kidney cells.

Conclusion: This study has generated new tools for the investigation of eukaryotic channels. The successful covalent linkage of four bacterial Nav channel monomers should permit the introduction of radial asymmetry into the structure of bacterial Nav channels and enable the known structures of these channels to be used to gain unique insights into structure-function relationships of their eukaryotic counterparts.

KEYWORDS: NaChBac, NavMs, NavAb, bacterial sodium channels, concatenation, patch clamp, immunodetection, Western blot

Background

Voltage-gated sodium channels (Na_vs) play fundamental roles in eukaryotes, including electrical signaling, secretion and synaptic transmission. These roles are highlighted in a wide range of diseases (e.g. periodic paralysis, arrhythmia, and epilepsy) which result from the malfunction of mammalian Na_vs. Eukaryotic Na_vs are large multi-subunit complexes [1]. The pore-forming subunit is composed of approximately 2,000 amino acid residues organized in to four domains, each domain comprising six transmembrane spanning (TMS) segments containing a voltage sensor (TMS S4) and a pore forming region (between TMS S5 and S6). Resolving the atomic structure of these proteins is essential for providing a molecular framework to enable elucidation of their function and disease mechanisms. However, their exceptional size and complexity has proved to be a major challenge. Consequently, there is only one report of resolving atomic-level (3.8 Å) structure for eukaryotic Na_vs [2] and with the caveat of the resolved channel lacking electrophysiological characterization. This current situation represents a significant gap in our understanding of the structure/function relationships of Na_vs.

The discovery of bacterial Na_vs has been important in addressing our lack of structural insight into eukaryotic Na_vs. Bacterial Na_vs are simplified homologues of eukaryotic Na_vs; their sequences are analogous to one domain (i.e. six TMS segments with a voltage sensor and a pore forming region) of a eukaryotic Na_v and they form functional

channels from homotetrameric assembly [3-8]. Their minimalist structure has enabled their atomic-level structures to be resolved, which together with their electrophysiological characterization and atomic simulations based on the resolved structures, have been pivotal in gaining detailed understanding of ion permeation and gating for Na_vs [e.g. 9, 10].

However, there are several limitations of these bacterial models for the understanding eucaryotic Na_v channels. Foremost is that (unlike eukaryotic Nav channels) bacterial channels display radial symmetry (a consequence of their homotetrameric structure). Consequently, the bacterial counterparts cannot be used to investigate experimentally the distinct role of the four individual domains of eukaryotic Na_v channels and as homotetramers, it is not possible to generate asymmetry in a bacterial Na_v channel. For example, Xia et al. [11] constructed a model of Na_vRh with the selectivity filter (SF) mutated from the radially-symmetrical glutamate ring (EEEE) to the asymmetric ring of DEKA (to mimic the SF in eukaryotic Na_v channels) and through MD simulations of Na^+ permeation proposed a model to explain Na^+/K^+ selectivity in mammalian Na_v channels. Currently the predictions of the simulation study cannot be experimentally tested.

In an attempt to address this problem and to gain further insights in to the molecular mechanisms of ion permeation in eukaryotic Na_v using their bacterial counterparts, we attempted to generate a concatenated bacterial Nav channel in which four monomer

subunits are covalently linked to form a single polypeptide (and thus mirroring the structure of their eukaryotic counterparts). It was envisaged that such a structure would enable targeted mutation of individual domains of the concatemer and thus permit experimental testing of bacterial channels exhibiting asymmetry in the pore of the bacterial channels (e.g. Xia et al. study). We report intact expression of NaChBac and NavMs and NavAb concatemers but that stable expression was dependent on the expression system employed. Surprisingly, NaChBac concatemer was inherently unstable. However, NavMs concatemers could be expressed intact in mammalian cells and were amenable to electrophysiological investigation using the patch clamp technique.

Methods

Generation of expression vectors. cDNA constructs encoding NaChBac (GenBank accession number BAB05220) and NavMs (GenBank accession number WP_011712479) bacterial sodium channels were synthesized by EPOCH Life Science (www.epochlifescience.com).

NaChBac#1 tetramer was generated by covalently linking four NaChBac monomers (translation stop codons omitted) using hydrophilic linkers containing 16 amino acids (DTQKETLNFGRSTLEI [12]); unique restriction enzyme sites (*EcoRV, SphI and AfeI*) were incorporated between each monomer and a C-terminal FLAG epitope was engineered immediately upstream of the tetramer stop codon, as illustrated in

Supplementary Figure 5; see Supplementary Figures file. NaChBac#1 tetramer was subcloned into the pTracer-CMV (Invitrogen) mammalian expression vector at the *EcoRI/XbaI* sites downstream of the constitutive cytomegalovirus (CMV) promoter. Details for the generation of the trimer, dimer and monomer forms of NaChBac#1 are given in Supplementary Information (see Supplementary Information file).

NaChBac#2, NavAb and NavMs tetramers were generated by covalently linking four identical monomers (translation stop codons omitted) using poly-glycine and the amino acid sequence corresponding to the bovine NCX1 to generate a 61-amino acid linker (GGGGGGGGGGGGGGGGGGGGSHVDHISAETEMEGEGNETGECTGSYYCK KGVILPIWEDEP [13]); unique restriction enzyme sites (*EcoRI, EcoRV and AfeI*) were incorporated between each monomer/linker and a 3xMyc epitope was engineered immediately upstream of the stop codon. Tetramers were subcloned into pcDNA4/HisMaxC mammalian expression vector (EPOCH Life Science) respectively at the *KpnI/XbaI* sites downstream of CMV promoter and in-frame with the Xpress tag, generating an N-terminal Xpress epitope (Supplementary Figure 5E; see Supplementary Figures file). NaChBac#2 and NavMs tetramers were also subcloned into pTracer-CMV vector downstream of cytomegalovirus (CMV) promoter respectively for electrophysiological analysis. To investigate the expression conditions of NachBac#2 tetramer in yeasts and *E. coli*, it was subcloned into the pYES2 yeast expression vector and the pTBX1 bacterial expression vector at sites of *KpnI/XbaI* and *NdeI/NruI* respectively as described in Supplementary Information (see Supplementary Information file). Plasmid DNA were amplified by DNA Midiprep Kit (Qiagen).

130

131 *Cell culture and transfection*. Chinese hamster ovary (CHO) and human embryonic
132 kidney (HEK293T) cells were maintained in DMEM high glucose with L-glutamine
133 (Lonza) supplemented with 10% Fetal Bovine Serum (Gibco) with addition of 50 U/ml
134 Penicillin and 50 µg/ml Streptomycin (Sigma) in a humidified incubator at 37 ^{0}C and
135 5% CO_2. To introduce the expression of sodium channel genes, 10 µl of *Trans*IT-LT1
136 reagent (Mirus) and 5µg of plasmid DNA were equilibrated separately in 250 µl of
137 UltraMEM Reduced Serum Media (Lonza) for 5min before combining together and
138 incubating at room temperature for another 20min. The reagent-plasmid mixture was
139 then added to the seeded cells in the 6-well plate dropwise followed by incubating in
140 the incubator overnight.
141 *Saccharomyces cerevisiae* strain of W303.1a (*MATa ade2-1 ura3-1 his3-11,15 trp1-1*
142 *leu2-3,112 can1-100*) was cultured at 30 ^{0}C and transformed by lithium acetate method
143 [14]; transformants were selected by growth on synthetic complete media without uracil
144 (SCM-ura; Formedium, UK). Competent *E. coli* strain (Rosetta^{TM} DE3; Novagen) was
145 cultured at 37 ^{0}C and transformed by heat shock at 42 ^{0}C for 30 sec; transformants were
146 selected by growth on lysogeny broth (LB) media containing ampicillin.
147

148 *Protein extraction.* Protein extraction from CHO and HEK293T cells was performed
149 18-24 hours after transfection. After washing three times with cold PBS buffer
150 containing PierceTM Protease Inhibitor (Thermo Scientific), cells were lysed with
151 RIPA buffer (Sigma) plus phenylmethylsulfonyl fluoride (PMSF) and protease

inhibitor on ice for 10 min. The cell lysate was scrapped and transferred to the pre-cooled Eppendorf tubes for collecting supernatant by centrifugation at 13,000g for 15min at 4 ^{0}C.

Protein extracted from overnight cultures of *S. cerevisiae* (SCM-ura but with glucose replaced with 2% galactose and 2% raffinose to induce protein expression) was conducted by treating yeasts with 2 M of lithium acetate (LiAc) for 5min and then 0.4 M of NaOH for 10 minutes at room temperature. Supernatant was tested after centrifugation at 13,000g for 15 minutes at 4 ^{o}C. Protein expression was induced in *E. coli* by culturing in LB containing 0.4 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) for 1 hour at 37 ^{o}C with shaking at 150rpm. After washing, bacteria were lysed with Y-PER^{TM} Yeast Protein Extraction Reagent according to manufacturer's instruction (Thermo Scientific) with addition of proteinase inhibitor for 20 minutes at room temperature. Supernatant after centrifugation at 13,000 g for 15 minutes was retained for analysis.

Western blotting. Proteins were separated using 10% resolving sodium lauryl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (unless otherwise stated) and transferred to the Hybond^{TM}-P PVDF membranes (GE Healthcare Amersham) before blocking with 5% milk. The blots were subsequently treated with primary mouse antibody according to manufacturer's instruction (Thermo Scientific: Anti-c-Myc monoclonal antibody, MA1-21316; Anti-Xpress monoclonal antibody, R910-25;

Sigma-Aldrich: monoclonal ANTI-FLAG® M2 antibody, F1804) at 4 ºC overnight followed by secondary antibody (rabbit anti-mouse HRP; Abcam, Ab6728) for 1 hour at room temperature. Blots were washed 4 times with 1× phosphate buffered saline (PBS) containing 0.1% tween20 buffer for 5 minutes after the incubation with either the primary or secondary antibody. Signals were developed by PierceTM ECL substrates (Thermo Scientific) and imaged by ChemiDoc^{TM} (BioRad).

Electrophysiology. Whole-cell patch clamp recordings were acquired with Axopatch 200 series amplifiers (Molecular Devices, Sunnyvale, USA). Signals were digitized using Digidata1322 (Molecular Devices, Sunnyvale, USA). Data were filtered at 1 or 2 kHz. All the experiments were performed at 20ºC. Patch pipettes were produced by a pipette puller (model 730, KOPF instrument, USA) from KIMAX melting point capillary tubes (34500-99; Kimble Company, USA). Pipettes had resistances between 2 and 4 MΩ after filling with intracellular solution. Shanks of the pipettes tip were coated with bee's wax to reduce pipette capacitance. For investigation of the bacterial Na_V concatemers, the pipette solution was (in mM) 110 Cs-$MetSO_3$, 20 NaCl, 2 $MgCl_2$, 5 EGTA, 10 HEPES and 2 NaOH (pH 7.2, adjusted with 2 mM NaOH) and the bath solution was (in mM) 140 Na-$MetSO_3$, 2 $CaCl_2$, 10 HEPES, 2 $MgCl_2$, 2 NaOH to pH 7.4 (adjusted with 2 mM NaOH). Data collection was initiated 3 mins after obtaining whole cell configuration to ensure complete equilibration of the pipette solution and cytosol. The bath solution was grounded using a 3 M KCl agar bridge;

194 liquid junction potential determined experimentally [15] agreed with that calculated
195 (using JPCalc program, Clampex, Axon Instruments, Inc.) and was negligible. Results
196 were analyzed using Clampfit 10.1 software (Molecular Devices) and OriginPro8
197 (OriginLab Corporation). Pooled data are presented as means ± SEM (*n*), where *n* is
198 the number of independent experiments.

199

200 **Results and Discussion**

201 NaChBac was chosen to be concatenated based on previous reports of successful
202 concatenation [13,16]. A cDNA was synthesized to form a coding sequence composed
203 of four NaChBac genes concatenated into one open reading frame (Supplementary
204 Figure 5; see Supplementary Figures file). This synthetic gene (referred to here after as
205 NaChBac#1 tetramer) was designed to encode a tetrameric oligomer containing four
206 identical domains corresponding to NaChBac channels (translational stop codon
207 removed) tethered together using a 16 amino acid hydrophilic linker derived from
208 *Xenopus* γ-globin gene (which has been used in the successful expression of K^+ channel
209 oligomers in mammalian cells [12]). Restriction sites were strategically placed to
210 enable the extraction of individual monomers in the generation of both dimer and trimer
211 constructs (Supplementary Figure 5; see Supplementary Figures file). A C-terminal
212 FLAG epitope was added to enable immunological detection. The DNA constructs were
213 subcloned into mammalian expression vectors for expression in CHO and HEK293T
214 cells for electrophysiological analysis (pTracer-CMV2) and for immunodetection

(pIRESneo). It was envisaged that the transfection of cells with the tetramer construct would co-opt the cells biosynthetic machinery into creating a functional Nav channel formed from a single polypeptide and thus with a pre-determined monomer composition.

Immuno-detection of the FLAG epitope and Western blot analyses revealed NaChBac monomer with an approximate (expected [17]) size of 30 kDa (Supplementary Figure 6; see Supplementary Figures file). A protein corresponding to approximately 130 kDa was expected for the intact expression of the NaChBac#1 tetramer; however, only smaller (faint) bands running at approximately 15 kDa (Supplementary Figure 6A, B; see Supplementary Figures file) were detected in both CHO and HEK cells. The size of the degradation products detected in Supplementary Figure 6 is consistent with both poor expression and cleavage of the concatemer channels corresponding to a site in the S5 TMS region in the C-terminal domain. A similar pattern of degradation was observed for dimer and trimer constructs (Supplementary Figure 6A, B). Immunostaining of transfected CHO cells expressing NaChBac#1 monomer and tetramer and confocal microscopy (Supplementary Information section 3 and Supp. Figure 7; See Supplementary Information file and Supplementary Figures files respectively) were indistinguishable and showed the FLAG epitope at the plasma membrane of cells. This indicated degradation fragments of the NaChBac oligomers to be present in the plasma membrane and raised the possibility that these “fragments” could interact to form functional channel [18]. To test this possibility, the patch clamp technique was employed to record whole cell plasma membrane ion channel activity in CHO cells

237 expressing NaChBac#1 oligomer proteins. Expression of the NaChBac#1 oligomers
238 produced whole cell currents with similar kinetic properties to that exhibited by cells
239 expressing the NaChBac monomer (Supplementary Figure 6C; see Supplementary
240 Figures file). Thus, oligomer degradation appears to generate lower-order by-products,
241 which associate to form functional channels. Consistent with this, cells expressing
242 NaChBac#1 trimer (which would not be expected to form independent functional
243 channels if intact [19]) also exhibited NaChBac-like whole cell currents
244 (Supplementary Figure 6C; see Supplementary Figures file). Formation of functional
245 channels following degradation of concatenated ATP-gated P2X channels [18] has also
246 been reported.

247 The failure to express intact NaChBac concatemers in CHO and HEK cells was
248 surprising. The 16 amino acid linker used to generate NaChBac#1 oligomers has
249 previously shown to stably concatenate K^+ channel monomers [12]; however, a
250 minimum length of linker has also been reported for successful concatenation of GABA
251 receptor subunits [20]. To address the possibility that the linker was too short and to
252 investigate the possibility that stable concatentation depended on the choice of
253 expression system and/or the choice of bacterial Na_v, alternative concatemers of
254 NaChBac, Na_vMs and Na_vAb were investigated as detailed below.

255

256 ***Generation and detection of NaChBac#2, NavMs and NavAb oligomers***

257 A second NaChBac tetramer was generated (referred to here after as NaChBac#2) using

a longer (61 amino acid) peptide linker composed of 20x glycine residues and a 41 amino acid linker corresponding to a partial sequence from bovine NCX1. A C-terminal 3xMyc epitope was included to enable immunodetection (see Supplementary Information file). This linker was chosen based on its use in a previous report in which intact NaChBac tetramers were reported in HEK293T cells [13], though a 20× polyglycine linker was also reported for the generation of NaChBac dimers and trimers in CHO [16] and HEK cells [13] respectively. To address the weak expression of degradation products observed in the expression of NaChBac#1 (Supplementary Figure 6; see Supplementary Figures file), the pcDNA4 expression vector was employed for immunodetection. This vector enables the inclusion of an N-terminal Xpress epitope and drives strong expression levels in mammalian cells due to the presence of a QBI SP163 element (a strong translational enhancer aimed at improving expression and detection levels).

Surprisingly, degradation of the NaChBac#2 oligomers was also evident after expression in HEK293T and CHO cells (Figure 1A and B). Note that the degradation products detected using either N-terminal Xpress tag or a C-terminal Myc tag corresponded to approximate cleavage in the S5 domain (equivalent to that seen for NaChBac#1 oligomer). It is also noteworthy that the strong expression of NaChBac monomer using pCDNA4 also suffers degradation (which is not apparent using more modest expression levels driven by pTracer-CMV). This is consistent with NaChBac being inherently unstable. To investigate the expression in non-mammalian cells, NaChBac#2 tetramer was also expressed in yeast (*Saccharomyces cerevisiae*; Figure

1C) and bacteria (*Escherichiacoli*; Figure 1D). Similar degradation of NaChBac#2 tetramer was detected in *E. coli* but intact NaChBac#2 tetramer was apparent in yeast albeit with detectable lower grade degradation. These results suggest that NaChBac oligomers are inherently unstable, though the intact expression in a yeast raises the possibility of using *Pichia pastoris* [21] for high-level protein production (which could be used in downstream biochemical and structural investigations of NaChBac oligomers).

The failure to generate intact NaChBac oligomers in CHO and HEK cells is at odds with previous report [13, 16]. However, Zhao et al. [16] employed only electrophysiological characterization (with no reported immunodetection) in the analysis of their NaChBac dimer construct expressed in CHO cells. Although the electrophysiological characterizations and subsequent interpretations by Zhao et al. are reasonable, this approach cannot exclude the possibility of lower grade degradation. Pavlov et al. [13] reported intact oligomer generation (in HEK cells) free from degradation using Western blot analysis (albeit also at lower expression levels to that reported for the monomer). This contrasts with the present study despite the use of the same linker and expression vector in HEK cells. A possible explanation of this discrepancy may lie in Pavlov et al. using a nickel column to isolate N-terminally HIS-tagged NaChBac prior to Western blotting which may have inadvertently selected for intact products.

To explore other bacterial voltage-gated sodium channels, equivalent NavAb and

NavMs oligomers were generated. NavAb expression in mammalian cells was relatively low (Supplementary Figure 8; see Supplementary Figures file) and difficult to detect consistently; consequently NavAb was not investigated further. Figure 2A shows the detection of Myc epitopes from total protein extracted from CHO cells expressing Na_vMs and indicating substantial degradation (note weak expression levels associated with degradation products). However, equivalent expression in HEK cells showed that Na_vMs oligomers remained intact with no detectable lower order degradation apparent (Figures 2B and C). Furthermore, confocal microscopy also showed that the Na_vMs tetramer was located on the plasma membrane (Supplementary Figure 9; see Supplementary Figures file). Based on these results, the Na_vMs tetramer was taken forward for electrophysiological investigation to establish functionality.

Electrophysiological properties of concatenated Na_vMs oligomers

Figure 3A shows typical whole cell currents from cells expressing monomeric, trimeric and tetrameric forms of Na_vMs. Notably, Na_vMs-like whole cell currents were detected in HEK cells expressing Na_vMs monomer and tetramer but not in cells expressing the trimeric form. This contrasted with Na_vMs trimer expression in CHO cells (which showed degradation of NavMs; Figure 2A) in which whole cell currents consistent with the NavMs oligomer degradation are detectable (Figure 3A (iv)). The absence of NavMs-mediated currents in HEK cells expressing the trimer constructs confirms the integrity of the concatemer approach to control subunit assembly in the formation of NavMs channels and is consistent with the conclusion that subunits from different

concatemers do not interact. Mean current density (Figure 3B) from cells expressing Na_vMs monomer (30.20±12.89 pA/pF; n = 7) was similar to that in cells expressing the Na_vMs tetramer (21.49±6.30 pA/pF; n = 6). Furthermore, fitting whole cell current with an exponential function revealed that the activation time constant (τ) was similar in cells expressing the monomer and tetramer (Figure 3C) and is similar to that previously reported for Na_vMs [22]. Taken together these results show that the NavMs oligomer is intact and exhibits equivalent electrophysiological characteristic to that for the Na_vMs monomer.

Conclusions

The covalent linkage of four bacterial Nav channel monomers resembles the macroscopic structure of their eukaryotic counterparts, which should enable the introduction of radial asymmetry into the structure of bacterial Nav channels. We have created a new tool for the investigation of Na^+ channels that will enable the physical construction and electrophysiological investigation of bacterial channels (with atomic-resolution structure) exhibiting a SF, for example, composed of an amino acid motif (DEKA) typical of eukaryotic Nav channels. For instance, Flood et al. [24] recently simulated Na^+ permeation in a simulation model of the human Nav1.2 channel constructed by grafting residues of its selectivity filter and external vestibule region onto a bacterial channel with atomic-resolution structure. Their simulations captured a Na^+ knock on conduction mechanism in which the DEKA ring lysine (in its protonated form) was seen to form a stable complex with carboxylates and Na^+. In contrast and in

the presence of K^+, the K^+-lysine-carboxylate complex is non-existent resulting in the lysine acting as an electrostatic plug blocking K^+ permeation. The finding that the NavMs bacterial channel can be stably concatenated opens up the possibility of experimentally testing these predictions/modelling results by physically constructing a bacterial channel chimera in which the human Nav1.2 selectivity and vestibule region is grafted onto the NavMs concatemer. A further use of the concatemer is in the testing of ion permeation models which propose that the charge (Q_f) associated with the amino acid residues forming the SF are the principal factor underlying ion selectivity [23]. In these studies, the selectivity filter of the concatamer can be mutated with the substitution of additional D or E residues to change the value of Q_f in steps of *-1e* (as opposed to steps of *-4e* when using the monomer) and thus provide a more detailed investigation of the role of Q_f enabling model development.

Declarations

Ethics approval and consent to participate. Not applicable.

Consent for publication. Not applicable.

Availability of data and material. The data that support the findings of this study are included in the published article and supplementary information and figures files. Further information is available from the corresponding author

Competing interests. The authors declare that they have no competing interests.

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manuscript.

Authors contributions. HS and ZZ planned and performed experiments to construct and validate (biochemical and electrophysiological) channel concatemers and assisted in preparation of manuscript. OF planned and performed electrophysiological characterisation of NaChBac and NavMs and assisted in writing the manuscript. SR conceived and designed the experiments, assisted in the generation of the channel constructs, interpreted the data and wrote the manuscript. All authors have read and approved the manuscript.

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Supplementary files. "Supplementary Information" file contains additional information on the construction of bacterial channel concatemers, immunolocalization methodology and description of results and supplementary figures 1 – 4 which show the DNA sequences of the channel concatemers. "Supplementary Figures" file contains Supplementary Table 1 listing the primers used for construction of the channel concatemers and Supplementary Figure 5 – 9 which show additional Western blot (and electrophysiological) analyses of channel expression and immunolocalization confocal images of channel constructs. All supplementary figures are referred to in the main text .

List of abbreviations

SCM: synthetic complete media

LB: lysogeny broth

PMSF: phenylmethylsulfonyl 19luoride

LiAC: lithium acetate

TMS: transmembrane spanning domains

Navs: voltage gated sodium channels

CMV: cytomegalovirus

ITPG: Isopropyl ß-D-1-thiogalactopyranoside

SDS PAGE: sodium lauryl sulfate polyacrylamide gel electrophoresis

PBS: phosphate buffer saline

Note that standard abbreviations for amino acids, nucleic acid and element symbols are used.

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FIGURE LEGENDS

Figure 1. Western blot analyses of total cell protein extracted from (A) CHO, (B) HEK293T, (C) yeast and (D) bacterial cells expressing NaChBac#2 oligomers. (A) Upper panel shows reaction to anti-Myc antibody; lower panel is loading control and shows reaction to anti-β-actin antibody. Empty vector (EV) control was the plasmid of pCDNA4. (B) Upper panels show reaction to anti-Xpress antibody; lower panel is loading control and shows reaction to anti-β-actin antibody. EV control was pCDNA4. (C) Upper panel shows reaction to anti-Myc antibody; lower panel is loading control and shows reaction to anti-GAPDH antibody. EV control was pYES2. (D) Upper panel shows reaction to anti-Myc antibody; lower panel is loading control and shows reaction to anti-GAPDH antibody. EV control was pTBX1.

Figure 2. Western blot analyses of total cell protein extracted from (A) CHO and (B and C) HEK293T cells expressing NavMs oligomers. Upper panels show reaction to anti-Myc or Xpress antibody; lower panels are loading control and show reaction to anti-β-actin antibody. EV control was the plasmid of pCDNA4. Arrows indicate expected sizes of intact oligomers.

Figure 3. Current density and kinetic properties of NavMs expressed in HEK293T cells. (A) Typical whole cell currents recorded from HEK293 cells transfected to express (i) monomeric, (ii) tetrameric and (iii) trimeric NavMs. (iv) Typical recording from CHO cells expressing trimeric NavMs. Currents were recorded in response to stepping the voltage from 40 mV to -60 mV in -20 mV step from a Vhold of -140 mV. (B) Mean peak current density from HEK293 cells expressing tetrameric (closed squares; n = 7), and monomeric (open squares; n = 6) NavMs. Error bars represent SEM. (C) Activation kinetic properties (determined from fitting an exponential power function). Currents result from Vhold of -140 mV). Error bars represent SEM.