Supplemental Information

Mapping of voltage sensor positions in resting and inactivated mammalian sodium channels by LRET

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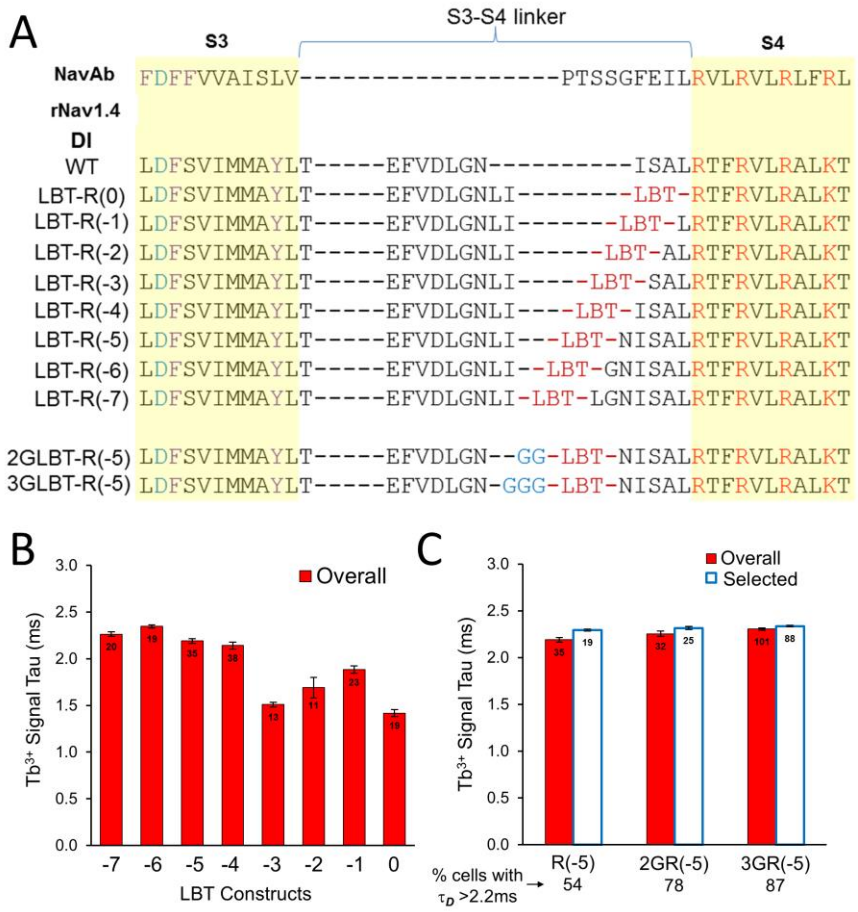
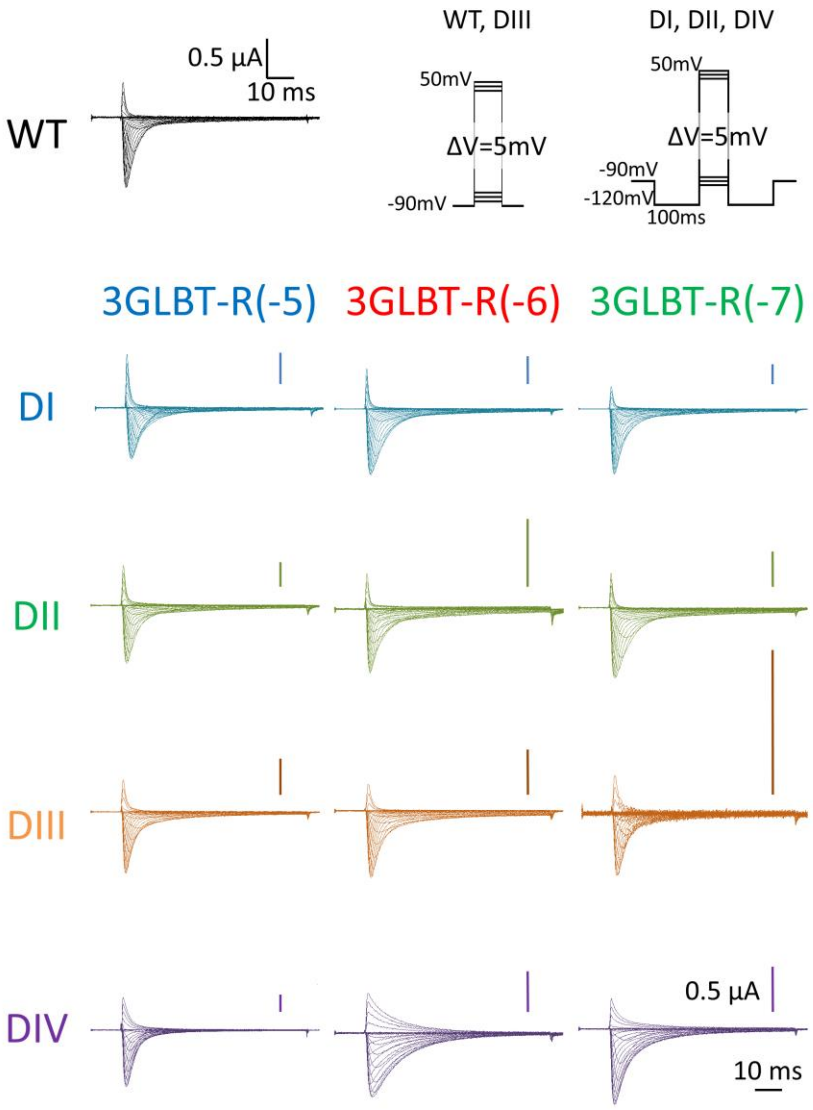
Supplemental Information



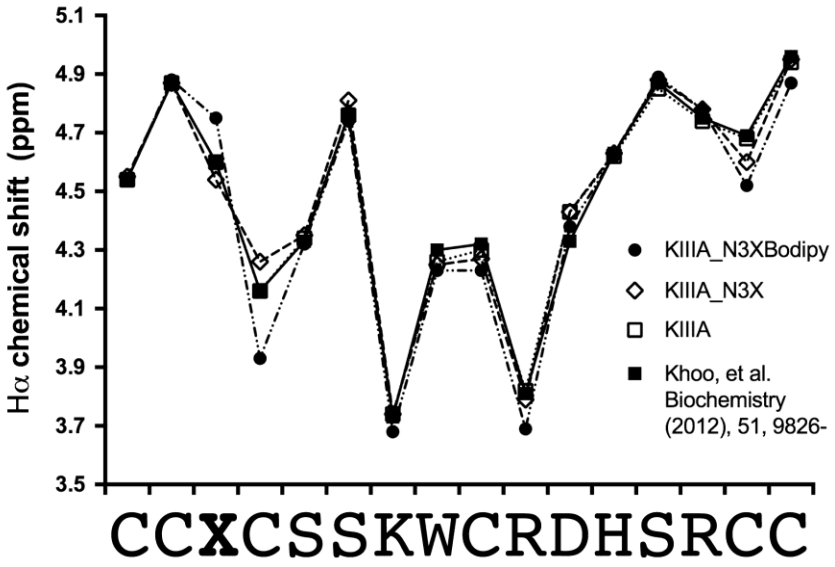
Supplemental Fig.S1 related to Fig.2. Scanning the optimal position of LBT insertion at the top of S4 in DI.

(A) Sequence alignment of all DI-LBT clones tested. Insertion of residues LI occurred during the subcloning process when the LBT was inserted. When DI-2GLBT-R(-5) and DI-3GLBT-R(-5) were generated, the LI insertion was replaced by glycines. (B) Time constant of ***D*** signal from eight different DI-LBT clones with the LBT in different positions. These data were collected from at least three different batches of oocytes. Figures inside the columns are the number of cells tested. Error-bars indicate SEM. (C) Time constant of ***D*** signals from DI-LBT-R(-5), DI-2GLBT-R(-5) and DI-3GLBT-R(-5). Red bars (overall) indicate the average values from all cells examined. The blue bars (selected) indicate the average values of cells selected from the whole set that showed time constants slower than 2.2 ms. The number of cells evaluated in the overall and selected groups are shown within the corresponding bars. The percentage of cells with time constants slower than 2.2 ms is shown at the bottom (R(-5) = 19/35, 2GR(-5) = 25/32 and 3GR(-5) = 88/101, respectively). Error bars indicate SEM.



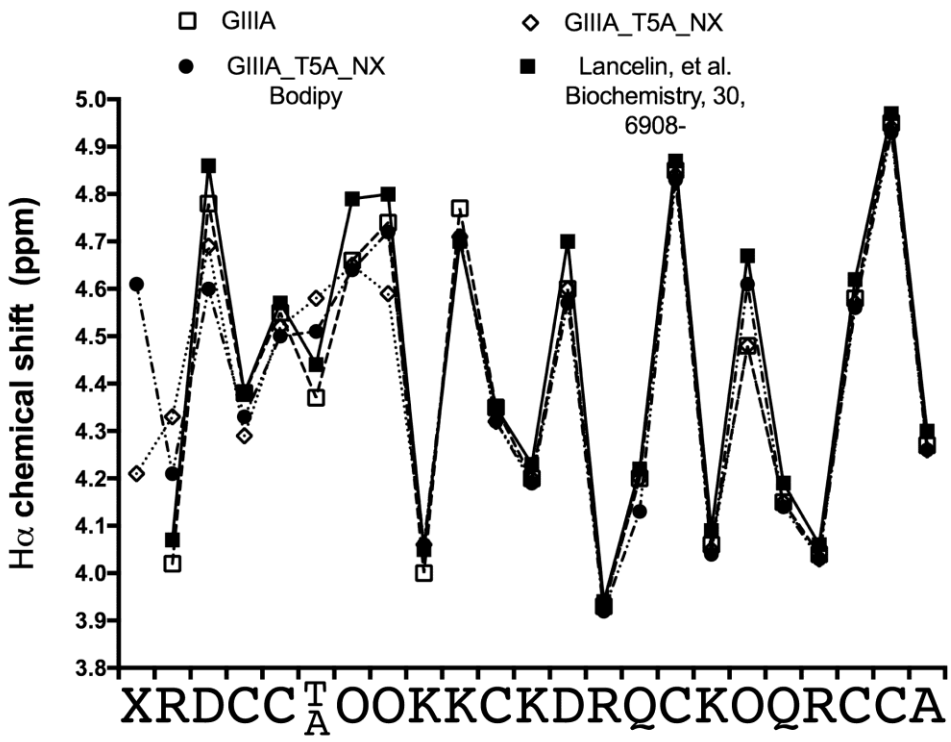
Supplemental Fig. S2 related to Fig.2. Representative Na^+ ionic current from Nav-LBTs.

Representative Na^+ ionic currents from wild type (WT) and all Nav-LBTs are shown. Right upper inset shows the pulse protocols used for the measurements. The protocol to the left was used for wild type (WT) and for DIII-LBTs recordings. The protocol to the right was used for DI-LBTs, DII-LBTs and DIV-LBTs. Horizontal and vertical scale bars throughout indicate 10 ms and 0.5µA, respectively. A small reversal potential shift is apparent for some families of traces shown. We attribute this variability to incomplete equilibration of Na concentration in the internal solution in the cut open oocyte within the time window chosen during the experiment. Slowing of fast inactivation was observed in DIV-3GLBT-R(-6) and (-7).

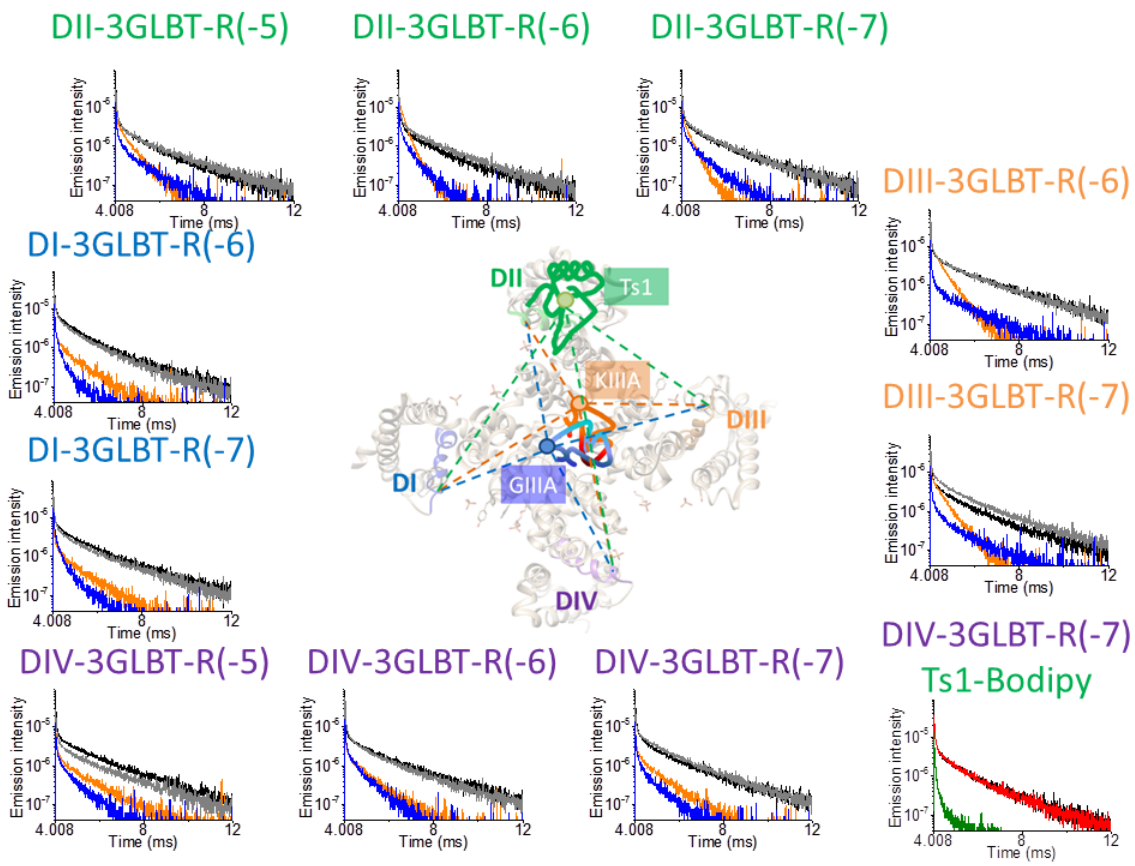


Supplemental Fig. S3 related to Fig.3. Hα-chemical shift analysis of synthetic KIIIA and analogues.

Despite minor differences near the site of modification (residue 3), the overall fold of all analogues is highly similar and in agreement with literature data reported previously.

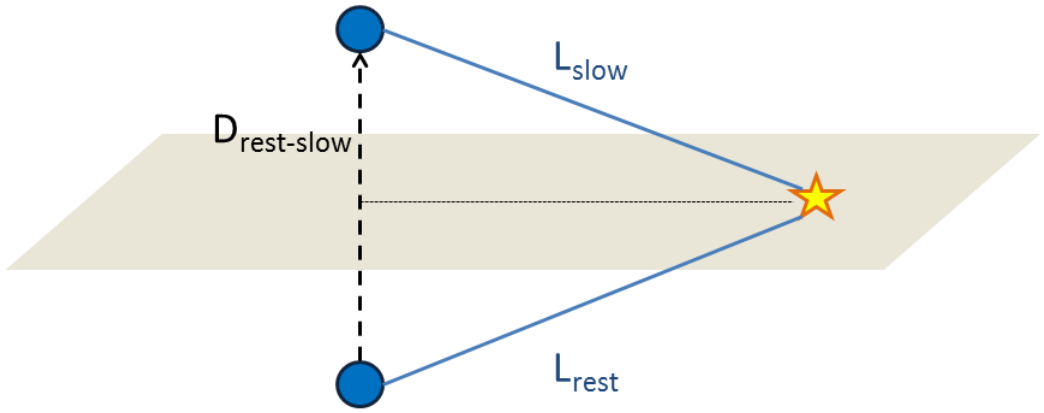


Supplemental Fig. S4 related to Fig.3. Hα-chemical shift analysis of synthetic GIIIA and analogues.



Supplemental Fig.S5 related to Fig.5. Representative LRET signals in all LBT-Navs using three different acceptors.

In the case of KIIIA-Bodipy as the acceptor, black traces indicate the donor (***D***) signal before applying the acceptor, and orange trace indicates the sensitized emission (***SE***) signal from an identical cell in the presence of 0.85 μM KIIIA-Bodipy. Similarly, in case of GIIIA-Bodipy as the acceptor, the gray trace represents the ***D*** signal and blue signal is ***SE*** signal from a separate cell in the presence of 0.85 μM GIIIA-Bodipy. In LRET signal from DIV-3GLBT-R(-7) using Ts1-Bodipy, the black trace indicates the ***D*** signal, the red one indicates the donor in the presence of acceptor (***DA***) signal, the and green one is the ***SE*** signal after pretreatment with Ts1-Bodipy.



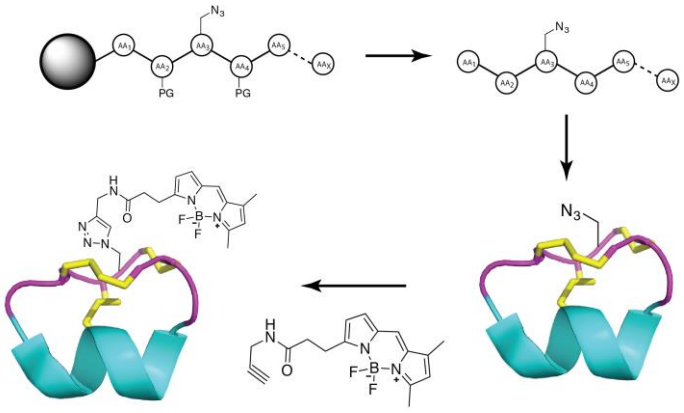
Supplemental Fig.S6 related to Fig.6 and Fig 7. Diagram illustrating why LRET measurements in this work may be insensitive to movements perpendicular to the membrane

The figure shows a case in which the donor moves perpendicular to the plane of the membrane (gray sheet), such that the distances between donor and acceptor are equal in the resting and slow inactivated states. Blue balls represent the positions of the donor, and the star represents the acceptor. Blue solid lines indicate the distance measured by LRET in the resting state (L_{rest}) and in the slow-inactivated state (L_{slow}). The black dashed line indicates the actual donor movement. In this extreme case, the LRET measurement will not detect a movement. If the donor moves between positions placed asymmetrically with respect to the acceptor, a distance change will be detected.

Supplemetal Experimental Procedures

Chemical synthesis of dye-conjugated peptide toxins

KIIIA, KIIIA-Bodipy and GIIIA-Bodipy:



Site-specific incorporation of Bodipy was achieved by click-conjugation of an azide-containing polypeptide precursor with Bodipy-alkyne (Lumiprobe, Germany). The β-azido alanine (X) building block was incorporated in position 3 of KIIIA or added to the N-terminus of GIIIA-T5A. A preliminary test of blocking activity by μCTX GIIIA-T5A was performed using two-microelectrode voltage clamp to record from rat Nav1.4 channels expressed in oocytes. The half-blocking concentration (IC_{50}) was estimated as follows using [GIIIA-T5A] = 10 nM:

$$IC_{50} = \{(1/fb)-1)\ [GIIIA\text{-}T5A]\},$$

where fraction blocked = fb = I_{tx}/I_o, and I_{tx} = peak I_{Na} in the presence of the GIIIA, and I_o = peak I_{Na} in the absence of the peptide. For GIIIA-T5A, IC_{50} (nM) = 9.8 ± 3.8 (mean ± s.d., n=5). For comparison, Shon et al (1998) reported a value of 6.3 nM for wildtype GIIIA in similar experiments.

The following peptides were chemically synthesized by Fmoc/tBu solid phase peptide synthesis on an Applied Biosystems automated synthesizer using Rink-amide resin: KIIIA (CCNCSSKWCRDHSRCC-NH2), KIIIA_N3X (CCXCSSKWCRDHSRCC-NH2) and GIIIA_N5A_NX (XRDCCAOOKKCKDRQCKOQRCCA-NH2; O = 4-hydroxyproline). Following chain assembly, peptides were cleaved from the solid support and side-chain deprotected using TFA:Triisopropylsilane:water (95:2.5:2.5 (v/v/v)) for 1.5 h at room temperature. The crude and fully reduced peptide was precipitated with diethylether, redissolved in acetonitrile (ACN):water (50:50) containing 0.1% TFA and lyophilized. The desired products were purified by RP-HPLC and lyophilized. ESI-MS for reduced peptides (average isotope composition) KIIIA Mcalc: 1890.2 Da, Mobsd: 1889.5 Da; KIIIA_N3X Mcalc: 1888.1 Da, Mobsd: 1887.4 Da; GIIIA_N5A_NX Mcalc: 2697.1 Da, Mobsd: 2696.7 Da).

Folding and formation of disulfides was carried out by first dissolving the peptide in 10% ACN/water at a concentration of 4 mg/mL and subsequent 1:40 dilution into folding buffer (100 mM Tris-HCl, 1 mM reduced glutathione, 1 mM oxidized glutathione, pH 7.5). The folding mixture reached equilibrium after 2h (HPLC), after which the solution was acidified with TFA to give a pH of 2-3, and the products were purified by preparative HPLC. In the case of KIIIA analogues, two closely eluting disulfide isomers were isolated, of which the earlier eluting isomer was identified by NMR spectroscopy as the desired analogue (disulfide connectivity I-V, II-IV, III-VI). MALDI-MS (most abundant isotope composition) KIIIA Mcalc: 1882.7 Da, Mobsd: 1882.6 Da; KIIIA_N3X Mcalc: 1880.6 Da, Mobsd: 1880.5 Da; GIIIA_N5A_NX Mcalc: 2689.1 Da, Mobsd: 2688.9 Da).

Click conjugation of the purified, azide-labeled conotoxins was performed in 50% ACN/water containing 3.5 mM peptide, 7 mM Bodipy-alkyne, 10 mM aminoguanidine, 20 mM ascorbate, 5 mM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methylamine (TBTA) and 5 mM CuSO4. The reaction reached completion within 2h after which the product was purified by RP-HPLC and isolated as an orange powder following lyophilization. MALDI-MS (most abundant isotope composition) KIIIA-Bodipy Mcalc: 2209.6 Da, Mobsd: 2209.5 Da; GIIIA-Bodipy Mcalc: 3018.2 Da, Mobsd: 3018.1 Da).

NMR spectroscopy was used to confirm the correct fold of the synthetic analogues. NMR experiments of all analogues were performed on a Bruker Avance 600 spectrometer equipped with a cryoprobe. Peptides were dissolved in H2O/D2O (9:1) at a concentration of 2-3 mg/mL. 1D 1H, 2D TOCSY, NOESY as well as 1H-13C HSQC spectra were recorded at 280 K (KIIIA) or 300 K (GIIIA) and used for sequential assignment of the spectra. The obtained Hα-chemical shifts provide a sensitive probe of peptide secondary and tertiary structure and were compared to literature values reported previously for wild-type KIIIA and GIIIA (Supplemental Fig. S3 and S4).

Supplemental Reference:

Shon KJ, Olivera BM, Watkins M, Jacobsen RB, Gray WR, Floresca CZ, Cruz LJ, Hillyard DR, Brink A, Terlau H, Yoshikami D. (1998) mu-Conotoxin PIIIA, a new peptide for discriminating among tetrodotoxin-sensitive Na channel subtypes. *J Neurosci* 18(12):4473-4481.