Published in final edited form as:
Neurosci Lett. 2009 October 25; 462(3): 272–275. doi:10.1016/j.neulet.2009.07.020.

The voltage-gated Na^+ channel β3 subunit does not mediate *trans* homophilic cell adhesion or associate with the cell adhesion molecule contactin

Dyke P. McEwen[1,2], **Chunling Chen**[1], **Laurence S. Meadows**[1,3], **Luis Lopez-Santiago**[1], and **Lori L. Isom**[1,*]
[1]Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109-0632

Abstract

Voltage-gated Na^+ channel (VGSC) β1 and β2 subunits are multifunctional, serving as both channel modulators and cell adhesion molecules (CAMs). The purpose of this study was to determine whether VGSC β3 subunits function as CAMs. The β3 extracellular domain is highly homologous to β1, suggesting that β3 may also be a functional CAM. We investigated the *trans* homophilic cell adhesive properties of β3, its association with the β1-interacting CAM contactin, as well as its ability to interact with the cytoskeletal protein ankyrin. Our results demonstrate that, unlike β1, β3 does not participate in *trans* homophilic cell-cell adhesion or associate with contactin. Further, β3 does not associate with $ankyrin_G$ in a heterologous system. Previous studies have shown that β3 interacts with the CAM neurofascin-186 but not with VGSC β1. Taken together, these findings suggest that, although β1 and β3 exhibit similar channel modulatory properties in heterologous systems, these subunits differ with regard to their homophilic and heterophilic CAM binding profiles.

Keywords

Na^+; channel; β subunit; cell adhesion molecule

Introduction

Voltage-gated ion channels are multi-functional [20]. In addition to regulating electrical excitability through ion conduction, some voltage-gated ion channels contribute to processes as diverse as intracellular signaling, transcriptional regulation, scaffolding, and cell adhesion without requiring changes in ion flux [5,6,24]. For example, VGSC β subunits regulate channel transcription, cell surface expression, and subcellular localization, modulate channel currents, and participate in cell-cell adhesion [4,8,14,17,22,25,31,33].

VGSCs isolated from mammalian neurons are heterotrimers, composed of a single α subunit, one non-covalently linked β subunit (β1 or β3), and one disulfide-linked β subunit (β2 or β4)

*To whom correspondence should be addressed: The University of Michigan, Department of Pharmacology, 1150 W. Medical Center Dr., 3422 Med Sci I, SPC 5632, Ann Arbor, MI 48109-5632, Tel.: 734-936-3050, Fax: 734-763-4450, E-mail: lisom@umich.edu.
[2]Present address: Essen Instruments, Ann Arbor, MI 48108
[3]Present address: Mathematics of Information Technology and Complex Systems, Simon Fraser University, Canada

[7,31]. VGSC α subunit cDNAs express functional channels in heterologous systems. However, for tetrodotoxin-sensitive α subunits, the currents characteristic of these channels expressed in isolation are different from native currents. Co-expression of β with α subunits in heterologous systems shifts the voltage-dependence of activation and inactivation, changes the rates of inactivation and recovery from inactivation, and increases channel cell surface expression [5,6,31]. VGSC β subunits and the β subunits of Ca^{2+} and K^+ channels are functionally homologous in terms of channel modulation [2,19]. However, Ca^{2+} and K^+ channel β subunits are not structurally homologous to VGSC β subunits. Of this group, only the VGSC β subunits contain extracellular Ig domains [18,19] and only the VGSC β1 and β2 subunits have been shown to function as cell adhesion molecules (CAMs) in addition to their roles in channel modulation [5,6]. An important question is whether all of the four known VGSC β subunits function as CAMs.

β1 and β2 function as CAMs: β1 and β2 interact with the extracellular matrix protein tenascin to influence cell migration [37,41]; β1 and β2 participate in *trans* homophilic cell-cell adhesion resulting in cellular aggregation and ankyrin recruitment [26,27]; the β1 extracellular Ig domain interacts with the neuronal and glial CAMs contactin, VGSC β2, neurofascin-155, neurofascin-186, and NrCAM, but interestingly not with VGSC β3 [21,29,30]; β1 interactions with contactin and neurofascin-186 result in increased channel cell surface expression [21, 29,30]; and β1 promotes neurite extension as a result of homophilic adhesion [4,12].

The purpose of the present study was to determine whether VGSC β3 subunits function as CAMs. The β3 extracellular domain is highly homologous to β1, suggesting that β3 may also be a functional CAM. Consistent with this hypothesis, β3 associates with the CAM neurofascin-186 [29,34]. We investigated the *trans* homophilic cell adhesive properties of β3, its association with the β1-interacting CAM contactin, as well as its ability to interact with the cytoskeletal protein ankyrin. Our results demonstrate that, unlike β1, β3 does not participate in *trans* homophilic cell-cell adhesion or associate with contactin. Further, β3 does not associate with $ankyrin_G$ in a heterologous system. These findings are relevant to recent findings showing that the *Scn3b* null phenotype is subtle and that the null mice are viable [15]. This is in contrast to the severe neurological phenotype of *Scn1b* null mice [8], supporting the hypothesis that physiological role of β1 *in vivo* may include both channel modulation and cell-cell adhesion, while the mechanism of β3 function may be less dependent on cell adhesion and more limited to channel modulation.

Materials and Methods

All experimental procedures are included in the on-line supplemental materials.

Results

β3-GFP modulates Na^+ currents expressed in Xenopus oocytes

To characterize the full scope of β3 function, we generated a GFP-tagged β3 plasmid, allowing for β3 detection using either β3 antiserum or a commercial anti-GFP antibody. We co-expressed β3-GFP with $Na_v1.2$ to determine if the addition of the epitope tag interfered with β3-mediated channel modulation. β3-GFP cRNA was coinjected with $Na_v1.2$ cRNA in *Xenopus* oocytes. As shown in Supplemental Fig. 1 and Supplemental Table 1, β3-GFP modulated Na^+ currents by shifting the half-voltage of inactivation in the hyperpolarizing direction, increasing the rate of inactivation, and increasing the peak current amplitude compared with currents expressed by $Na_v1.2$ alone ($Na_v1.2$: −1035 nA, $Na_v1.2$ + β3-GFP: −2160 nA), similar to previous reports with untagged β3 [32, 35]. Thus, we used β3 and β3-GFP interchangeably in all subsequent experiments.

β3 does not participate in trans homophilic cell-cell adhesion

We used *Drosophila* S2 cells previously to demonstrate that VGSC β1 and β2 subunits function in *trans* homophilic cell-cell adhesion resulting in ankyrin recruitment [26,27]. S2 cells are ideal for this type of experiment, as they are free-floating in suspension culture, and express no endogenous CAMs. cDNAs encoding putative CAMs to be tested are transfected into S2 cells under an inducible promoter and clonal lines established. Induction of protein expression followed by mechanical shaking allows a detailed analysis of the time course of cellular aggregation. To investigate the *trans* homophilic cell adhesive properties of β3, we expressed β3 or β3-GFP in *Drosophila* S2 cells and established stable, clonal cell lines using soft agar cloning techniques as in [26]. We verified expression of β3 or β3-GFP in these clonal lines by Western blot analysis with anti-β3 or anti-GFP, respectively (Fig. 1A, anti-β3; anti-GFP data not shown). Immunocytochemical analysis of S2-β3 and S2-β3-GFP cell lines using anti-β3 or anti-GFP antibodies showed robust β3 expression at the cell surface (Fig. 1B, anti-β3; anti-GFP data not shown). In spite of this expression, however, we were not able to detect β3-mediated cellular aggregation under any condition, including increasing the cell density in the assay, indicating that unlike β1, β3 does not mediate *trans* homophilic cell adhesion in S2 cells. In parallel experiments and under the same conditions, S2-β1 cells exhibited efficient aggregation as described previously [26,27] (data not shown), ensuring that the cell density in the experiment was sufficient to allow aggregation to occur.

β3 does not interact with contactin

We next asked whether β3 could interact with the neuronal and glial CAM contactin, as shown for β1 [21,29,30]. CHL cells stably expressing contactin were stably cotransfected with β1, β2, or β3-GFP. Triton X-100 solubilized cell lysates were immunoprecipitated with either non-immune IgG or anti-β-subunit antibodies. Contactin could be co-immunoprecipitated only from cells expressing β1 but not β2, in agreement with previous results [30], or β3-GFP (Fig. 2A). To determine whether the presence of a VGSC α subunit altered the ability of β3 to associate with contactin, we immunoprecipitated solubilized cells expressing $Na_v1.2$, β3, and contactin (Fig. 2B). While β3-GFP could be immunoprecipitated with anti-β3, anti-contactin was not able to precipitate β3, demonstrating that β3 and contactin do not associate either in the presence or absence of the ion-conducting pore. While these data demonstrated that β3 and contactin do not associate in a heterologous system, they did not indicate whether β3 could associate with a contactin-containing complex in brain, where many other channel-associating proteins are present [5,6]. To address this question, adult rat brain membrane preparations were solubilized in Triton X-100 and immunoprecipitated with either non-immune IgG, anti-contactin, or anti-β3, as indicated. The anti-β3 immunoprecipitate was positive for contactin (Fig. 2C), suggesting that, while β3 and contactin likely do not associate directly, a β3-contactin containing complex exists in brain. We postulate that the most likely β3 binding partner in brain is neurofascin-186, a CAM localized with the VGSC complex at nodes of Ranvier and axon initial segments that associates with both β1 and β3 subunits as well as ankyrin [10,11, 16,34].

β3 does not interact with $ankyrin_G$

β1 and β2 recruit ankyrin to points of cell-cell contact in response to *trans* homophilic cell-cell adhesion [26]. The interaction of β1 with ankyrin is dependent on the intracellular tyrosine residue Y181 [27]. Yeast-two-hybrid constructs generating the intracellular domain of β1 do not interact with constructs generating ankyrin [3,23], suggesting that extracellular homophilic β1-β1 adhesion is required for intracellular signaling. The intracellular domain of β3 contains a tyrosine residue at the position corresponding to β1Y181 (β3Y174), suggesting that β3 may also interact with ankyrin. However, if homophilic cell-cell adhesion is required to signal intracellular ankyrin recruitment by VGSC β subunits, then this interaction with β3 may not

take place. To test this, CHL cells were transiently transfected with either β1-GFP or β3-GFP and ankyrin$_G$-GFP (Fig. 3). Cell homogenates from confluent dishes in which all cells were in contact were solubilized in Triton X-100 and immunoprecipitated either with nonimmune IgG or anti-ankyrin$_G$. Blots were probed with anti-GFP to detect either β1-GFP or β3-GFP (Fig. 3, arrow). In agreement with previous results, ankyrin$_G$ associated with β1 [30]. These results also demonstrated that the presence of the GFP epitope tag on the carboxyl terminus of β1 did not disrupt its ability to interact with ankyrin$_G$. Under the same conditions, β3 did not associate with ankyrin$_G$ despite robust cellular expression levels. Taken together, these results suggest that *trans* homophilic cell-cell adhesion is required for VGSC β subunit-mediated ankyrin recruitment. Further, that β1 and β3 differ significantly in their signaling properties when expressed in heterologous systems.

Discussion

In spite of its similarity to β1, β3 does not exhibit *trans* homophilic cell adhesive interactions. The crystal structure of the extracellular domain of MP$_o$ [28,36] has been used previously to predict structure-function relationships for VGSC β1 subunits [28,36]. Comparison of the β3 Ig domain with the Ig domains of β1, β2, and MP$_o$ reveals a number of important amino acid differences that may explain the absence of β3 *trans* homophilic adhesive activity (Fig. 4). Four proline residues are present in the β3 Ig loop domain that are not present in the corresponding positions of the other three molecules: β3P16, located in the A'-B connecting loop of the Ig domain; β3P63, located in the C"-D connecting loop of the Ig domain; β3P109, located in F-G connecting loop of the Ig domain; and β3P118, located within β sheet G. P16, P63, and P109 are positioned in previously described flexible loop segments connecting the β sheets [38,40]. The presence of prolines in these regions may add rigidity to the flexible loops of the β3 Ig domain, limiting its ability to interact with other CAMs. A MP$_o$ mutation found in Charcot-Marie-Tooth patients, S49L, is located in the putative homophilic adhesive interface of MP$_o$ [36,39]. Alignment of this region of MP$_o$ with the corresponding regions of β1, β2, and β3 shows a lysine residue at this position in β1, a methionine residue in β2, and the absence of a corresponding residue in β3 (Fig. 4, "-"). If MP$_o$S49 is critical for adhesive interactions, then the lack of a corresponding residue at this position in β3 may contribute to its inability to participate in *trans* homophilic adhesive interactions.

In summary, these data demonstrate that, in spite of its high degree of similarity to β1, β3 does not mediate *trans* homophilic cell adhesion resulting in ankyrin recruitment. While β1 and β3 both bind to the CAM neurofascin-186 [30,34], they do not bind to each other [29], and of the two, only β1 binds to the CAM contactin [21,29,30]. Taken together, these results support the idea that, in spite of similar channel modulatory properties, the structures of the β1 and β3 Ig loop domains are significantly different and their ability to transduce intracellular signals differ as well. This hypothesis is supported by results showing that *Scn1b*, encoding β1, but not *Scn3b*, encoding β3, is expressed in cancer cells where it modulates cell adhesion and migration [9]. In contrast, *Scn3b*, unlike *Scn1b*, is up-regulated in response to DNA damage and mediates a p53-dependent apoptotic pathway [1]. Thus, these two VGSC β subunits may play very different physiological roles *in vivo*. Finally, the present results in heterologous systems may shed light on recent data showing that that phenotype of *Scn3b* null mice is mild compared to *Scn1b* null mice [15]. It is possible that *Scn1b* compensates for *Scn3b* deletion but not vice-versa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Adachi K, Toyota M, Sasaki Y, Yamashita T, Ishida S, Ohe-Toyota M, Maruyama R, Hinoda Y, Saito T, Imai K, Kudo R, Tokino T. Identification of SCN3B as a novel p53-inducible proapoptotic gene. Oncogene 2004;23:7791–7798. [PubMed: 15334053]
2. Adelman JP. Proteins that interact with the pore-forming subunits of voltage-gated ion channels. Curr Opin Neurobiol 1995;5:286–295. [PubMed: 7580150]
3. Bouzidi M, Tricaud N, Giraud P, Kordeli E, Caillol G, Deleuze C, Couraud F, Alcaraz G. Interaction of the Nav1.2a subunit of the voltage-dependent sodium channel with nodal ankyrinG. In vitro mapping of the interacting domains and association in synaptosomes. J Biol Chem 2002;277:28996–29004. [PubMed: 12036953]
4. Brackenbury WJ, Davis TH, Chen C, Slat EA, Detrow MJ, Dickendesher TL, Ranscht B, Isom LL. Voltage-gated Na+ channel beta1 subunit-mediated neurite outgrowth requires Fyn kinase and contributes to postnatal CNS development in vivo. J Neurosci 2008;28:3246–3256. [PubMed: 18354028]
5. Brackenbury WJ, Djamgoz MB, Isom LL. An emerging role for voltage-gated Na+ channels in cellular migration: regulation of central nervous system development and potentiation of invasive cancers. Neuroscientist 2008;14:571–583. [PubMed: 18940784]
6. Brackenbury WJ, Isom LL. Voltage-gated Na+ channels: potential for beta subunits as therapeutic targets. Expert Opin Ther Targets 2008;12:1191–1203. [PubMed: 18694383]
7. Catterall WA. Cellular and molecular biology of voltage-gated sodium channels. Physiological Reviews 1992;72:S15–S48. [PubMed: 1332090]
8. Chen C, Westenbroek RE, Xu X, Edwards CA, Sorenson DR, Chen Y, McEwen DP, O'Malley HA, Bharucha V, Meadows LS, Knudsen GA, Vilaythong A, Noebels JL, Saunders TL, Scheuer T, Shrager P, Catterall WA, Isom LL. Mice lacking sodium channel beta1 subunits display defects in neuronal excitability,sodium channel expression, and nodal architecture. J Neurosci 2004;24:4030–4042. [PubMed: 15102918]
9. Chioni AM, Brackenbury WJ, Calhoun JD, Isom LL, Djamgoz MB. A novel adhesion molecule in human breast cancer cells: voltage-gated Na+ channel beta1 subunit. Int J Biochem Cell Biol 2009;41:1216–1227. [PubMed: 19041953]
10. Davis JQ, Bennett V. Ankyrin binding activity shared by the neurofascin/L1/NrCAM family of nervous system cell adhesion molecules. J Biol Chem 1994;269:27163–27166. [PubMed: 7961622]
11. Davis JQ, Lambert S, Bennett V. Molecular composition of the node of ranvier: identification of ankyrin- binding cell adhesion molecules neurofascin (mucin+/third FNIII domain-) and NrCAM at nodal axon segments. J. Cell Biol 1996;135:1355–1367. [PubMed: 8947556]
12. Davis TH, Chen C, Isom LL. Sodium Channel β1 Subunits Promote Neurite Outgrowth In Cerebellar Granule Neurons. J. Biol. Chem 2004;279:51424–51432. [PubMed: 15452131]
13. Fein AJ, Meadows LS, Chen C, Slat EA, Isom LL. Cloning and expression of a zebrafish SCN1B ortholog and identification of a species-specific splice variant. BMC Genomics 2007;8:226. [PubMed: 17623064]
14. Fein AJ, Wright MA, Slat EA, Ribera AB, Isom LL. scn1bb, a zebrafish ortholog of SCN1B expressed in excitable and nonexcitable cells, affects motor neuron axon morphology and touch sensitivity. J Neurosci 2008;28:12510–12522. [PubMed: 19020043]
15. Hakim P, Gurung IS, Pedersen TH, Thresher R, Brice N, Lawrence J, Grace AA, Huang CL. Scn3b knockout mice exhibit abnormal ventricular electrophysiological properties. Prog Biophys Mol Biol 2008;98:251–266. [PubMed: 19351516]
16. Hedstrom KL, Rasband MN. Intrinsic and extrinsic determinants of ion channel localization in neurons. J Neurochem 2006;98:1345–1352. [PubMed: 16787401]
17. Isom LL. The role of sodium channels in cell adhesion. Front Biosci 2002;7:12–23. [PubMed: 11779698]
18. Isom LL, Catterall WA. Na^+ channel subunits and Ig domains. Nature 1996;383:307–308. [PubMed: 8848042]
19. Isom LL, De Jongh KS, Catterall WA. Auxiliary subunits of voltage-gated ion channels. Neuron 1994;12:1183–1194. [PubMed: 7516685]

20. Kaczmarek LK. Non-conducting functions of voltage-gated ion channels. Nat Rev Neurosci 2006;7:761–771. [PubMed: 16988652]
21. Kazarinova-Noyes K, Malhotra JD, McEwen DP, Mattei LN, Berglund EO, Ranscht B, Levinson SR, Schachner M, Shrager P, Isom LL, Xiao Z-C. Contactin associates with Na^+ channels and increases their functional expression. J. Neurosci 2001;21:7517–7525. [PubMed: 11567041]
22. Kim DY, Carey BW, Wang H, Ingano LA, Binshtok AM, Wertz MH, Pettingell WH, He P, Lee VM, Woolf CJ, Kovacs DM. BACE1 regulates voltage-gated sodium channels and neuronal activity. Nat Cell Biol 2007;9:755–764. [PubMed: 17576410]
23. Lemaillet G, Walker B, Lambert S. Identification of a conserved ankyrin-binding motif in the family of sodium channel alpha subunits. J Biol Chem 2003;278:27333–27339. [PubMed: 12716895]
24. Levitan IB. Signaling protein complexes associated with neuronal ion channels. Nat Neurosci 2006;9:305–310. [PubMed: 16498425]
25. Lopez-Santiago LF, Meadows LS, Ernst SJ, Chen C, Malhotra JD, McEwen DP, Speelman A, Noebels JL, Maier SK, Lopatin AN, Isom LL. Sodium channel Scn1b null mice exhibit prolonged QT and RR intervals. J Mol Cell Cardiol 2007;43:636–647. [PubMed: 17884088]
26. Malhotra JD, Kazen-Gillespie K, Hortsch M, Isom LL. Sodium channel β subunits mediate homophilic cell adhesion and recruit ankyrin to points of cell-cell contact. J. Biol. Chem 2000;275:11383–11388. [PubMed: 10753953]
27. Malhotra JD, Koopmann MC, Kazen-Gillespie KA, Fettman N, Hortsch M, Isom LL. Structural requirements for interaction of sodium channel β1 subunits with ankyrin. J Biol Chem 2002;277:26681–26688. [PubMed: 11997395]
28. McCormick KA, Isom LL, Ragsdale D, Smith D, Scheuer T, Catterall WA. Molecular determinants of Na^+ channel function in the extracellular domain of the β1 subunit. J. Biol. Chem 1998;273:3954–3962. [PubMed: 9461582]
29. McEwen DP, Isom LL. Heterophilic interactions of sodium channel beta 1 subunits with axonal and glial cell adhesion molecules. J Biol Chem 2004;279:52744–52752. [PubMed: 15466474]
30. McEwen DP, Meadows LS, Chen C, Thyagarajan V, Isom LL. Sodium channel β1 subunit-mediated modulation of Nav1.2 currents and cell surface density is dependent on interactions with contactin and ankyrin. J Biol Chem 2004;279:16044–16049. [PubMed: 14761957]
31. Meadows LS, Isom LL. Sodium channels as macromolecular complexes: Implications for inherited arrhythmia syndromes. Cardiovasc Res 2005;67:448–458. [PubMed: 15919069]
32. Morgan K, Stevens EB, Shah B, Cox PJ, Dixon AK, Lee K, Pinnock RD, Hughes J, Richardson PJ, Mizuguchi K, Jackson AP. β3: An additional auxiliary subunit of the voltage-sensitive sodium channel that modulates channel gating with distinct kinetics. Proc. Natl. Acad. Sci. U.S.A 2000;97:2308–2313. [PubMed: 10688874]
33. O'Malley HA, Shreiner AB, Chen GH, Huffnagle GB, Isom LL. Loss of Na+ channel beta2 subunits is neuroprotective in a mouse model of multiple sclerosis. Mol Cell Neurosci 2009;40:143–155. [PubMed: 19013247]
34. Ratcliffe CF, Westenbroek RE, Curtis R, Catterall WA. Sodium channel beta1 and beta3 subunits associate with neurofascin through their extracellular immunoglobulin-like domain. J. Cell Biol 2001;154:427–434. [PubMed: 11470829]
35. Shah BS, Stevens EB, Pinnock RD, Dixon AK, Lee K. Developmental expression of the novel voltage-gated sodium channel auxiliary subunit beta3, in rat CNS. J Physiol 2001;534:763–776. [PubMed: 11483707]
36. Shapiro L, Doyle JP, Hansley P, Colman DR, Hendrikson WA. Crystal structure of the extracellular domain from P_o, the major structural protein of peripheral nerve myelin. Neuron 1996;17:435–449. [PubMed: 8816707]
37. Srinivasan J, Schachner M, Catterall WA. Interaction of voltage-gated sodium channels with the extracellular matrix molecules tenascin-C and tenascin-R. Proc. Natl. Acad. Sci. U. S. A 1998;95:15753–15757. [PubMed: 9861042]
38. Vaughn DE, Bjorkman PJ. The (Greek) Key to Structures of Neural Adhesion Molecules. Neuron 1996;16:261–273. [PubMed: 8789942]

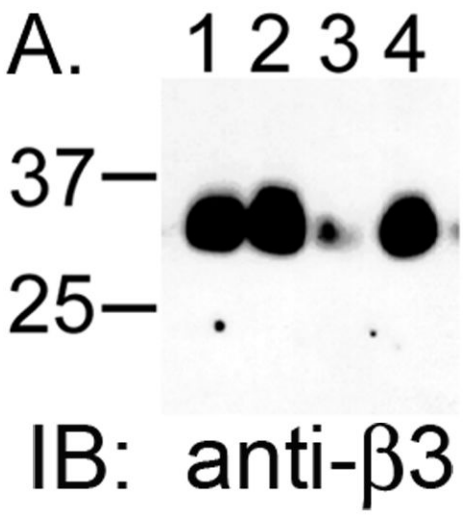
39. Warner LE, Hilz MJ, Appel SH, Killian JM, Kolodny EH, Karpati G, Carpenter S, et al. Clincal Phenotypes of different *MPZ* (P_O) mutations may include Charcot-Marie-tooth-type 1B, Dejerine-Sottas, and congenital hypomyelination. Neuron 1996;17:451–460. [PubMed: 8816708]

40. Williams AF, Barclay AN. The immunoglobulin superfamily--domains for cell surface recognition. Annu Rev Immunol 1988;6:381–405. [PubMed: 3289571]

41. Xiao Z-C, Ragsdale DS, Malhorta JD, Mattei LN, Braun PE, Schachner M, Isom LL. Tenascin-R is a functional modulator of sodium channel β subunits. J. Biol. Chem 1999;274:26511–26517. [PubMed: 10473612]

Acknowledgments

This work was supported by NIH R01MH59980 to LLI. DPM was supported by University of Michigan NIH training grant GM07767 and by an individual NRSA predoctoral fellowship NS43067.



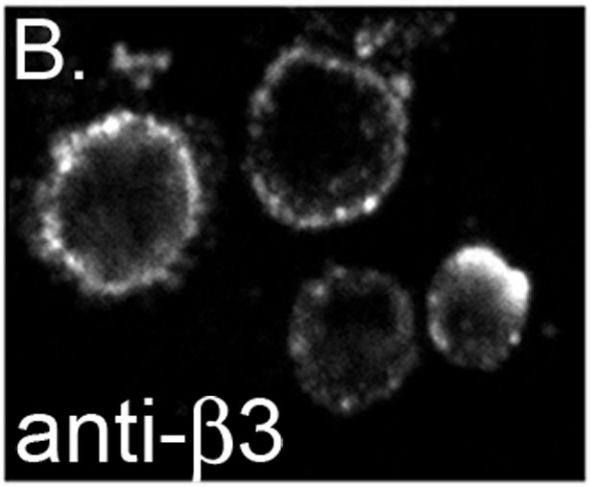


Fig. 1. β3 lacks *trans* homophilic cell adhesive properties

(A), Western blot analysis of *Drosophila* Schneider's line 2 (S2) cells transfected with β3, followed by soft agar cloning to select for stable clones. Protein expression was induced with 0.7 mM $CuSO_4$ for at least 24 h. Immunoblots (IB) were probed with anti-β3 antibody (1:500). Four representative clones are shown, three expressing high levels of β3 (lanes 1, 2, and 4). (B), Immunocytochemical analysis of β3 expression in S2 cells. Cells were fixed and stained with anti-β3 antibody (1:50), showing that β3 is expressed at the cell surface. None of the S2β3 cell clones aggregated following induction of protein expression and mechanical shaking. Panel shows a representative example of a high-expressing β3 cell line.

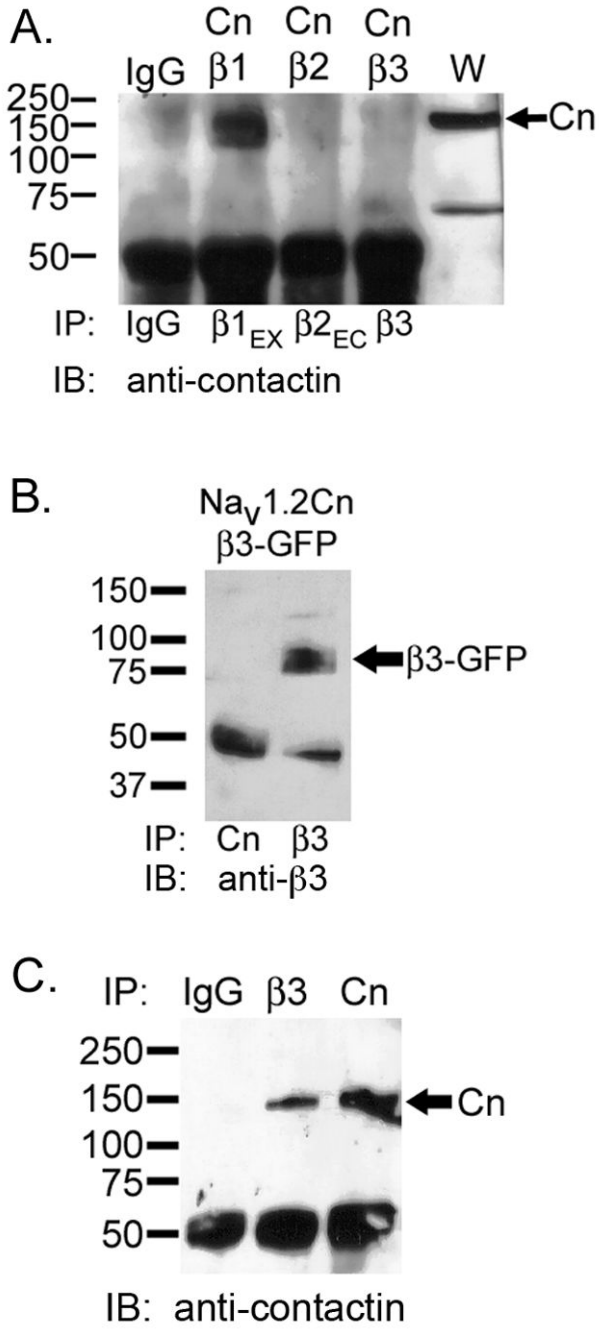


Fig. 2. β3 does not associate with contactin

(A), Immunoprecipitation from CHL cells stably expressing contactin and either β1, β2, or β3, as indicated. Triton X-100 solubilized lysates were immunoprecipitated (IP) with non-immune IgG, anti-$\beta1_{EX}$, anti-$\beta2_{EC}$, or anti-β3, as indicated, and the immunoblot (IB) was probed with anti-contactin antibody (1:500). 20 μg of rat brain lysate were used for positive contactin staining (W). Arrow indicates contactin (Cn) immunoreactive band. The bands at 50 kDa correspond to the antibody used for immunoprecipitation. (B), Immunoprecipitation from $Na_v1.2$/Cn/β3-GFP expressing cells. Triton X-100 solubilized lysates were immunoprecipitated (IP) with either anti-contactin antibody (lane 1) or anti-β3 antibody (lane

2) and the immunoblot (IB) was probed with anti-β3 (1:500). The ~30 kDa increase in the molecular weight of β3 (arrow) is due to the presence of the GFP tag. The lower bands at 50 kDa correspond to the antibodies used for immunoprecipitation. (C), Triton X-100 solubilized rat brain lysates were immunoprecipitated (IP) with nonimmune IgG, anti-β3, or anti-contactin antibodies, as indicated. Immunoblotting (IB) with anti-contactin yielded a band at 135 kDa in lanes 2 and 3 (arrow). Equal amounts of lysate were used for each immunoprecipitation. "Cn": contactin.

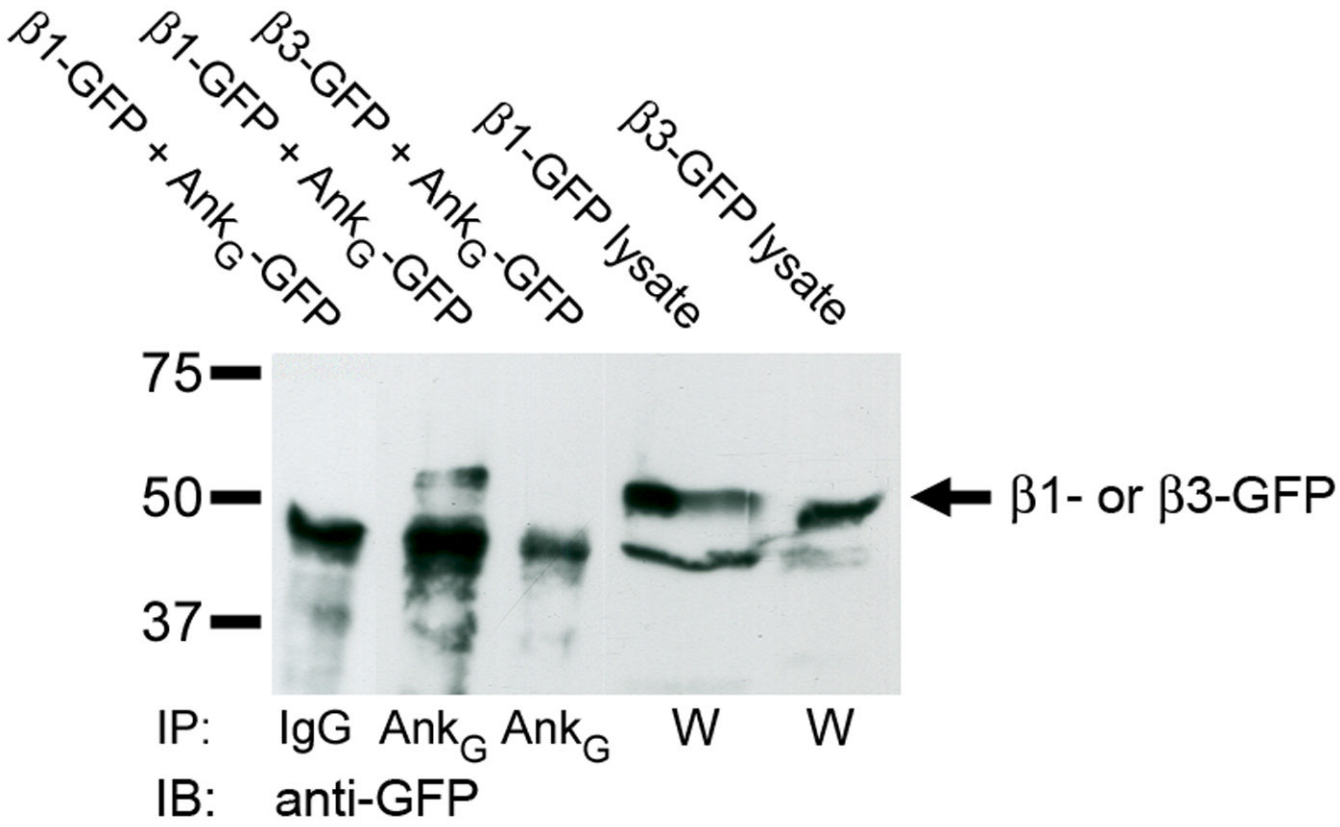


Fig. 3. β3 does not interact with ankyrin$_G$

Immunoprecipitation of β1-GFP or β3-GFP with ankyrin$_G$-GFP from transiently transfected CHL cells, as indicated. Equal amounts of Triton X-100 solubilized lysates of cells expressing the indicated combinations of β subunits and ankyrin$_G$ were immunoprecipitated (IP) with 5 μL of either non-immune IgG or anti-ankyrin$_G$, as indicated. Immunoblots (IB) were probed with anti-GFP antibody (1:500). Immunoreactive bands at approximately 60 kDa represent β1-GFP or β3-GFP, respectively (arrow). The increase in molecular weight of the β subunit immunoreactive bands is due to the addition of the GFP tag.

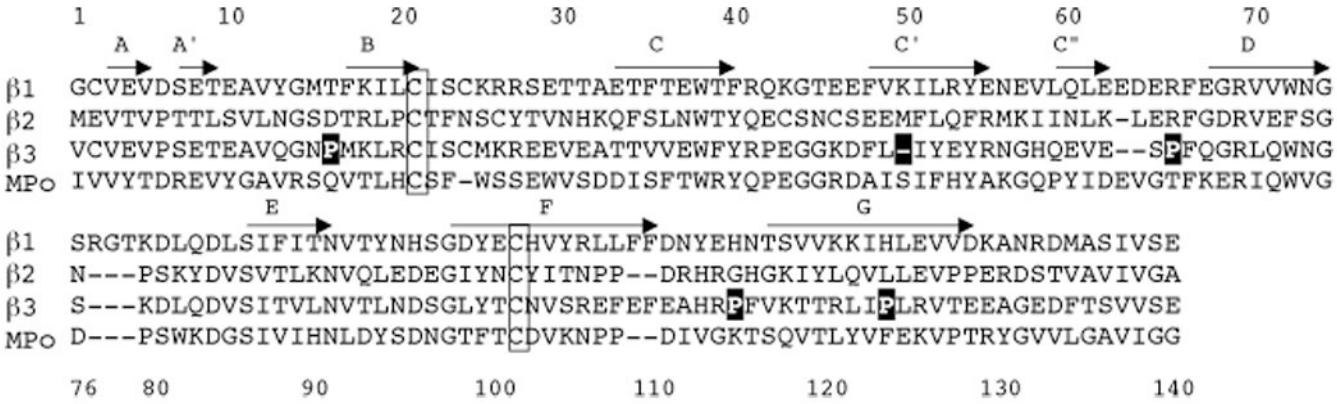


Fig. 4. Sequence alignment of the extracellular domains of β1, β2, β3, and MP_0

Amino acid sequences for the β1, β2, β3, and MP_0 extracellular domains were acquired from NCBI (β1 accession number: M91808; β2: NM_012877; β3: AF378093; MP_0: NM_017027) and aligned using the program MSA (http://xylian.igh.cnrs.fr/msa/msa.html). Numbering corresponds to the β1 amino acid sequence. Arrows indicate positions of β sheets in the Ig domain, as in [13]. β1 and β3 share ~43% sequence identity. Cysteines involved in formation of the Ig loop are shown in clear boxes. Proline residues unique to β3 are highlighted in black.