Published in final edited form as:

Neuropharmacology. 2018 April ; 132: 43–57. doi:10.1016/j.neuropharm.2017.09.018.

Voltage-gated sodium channel β subunits: The power outside the pore in brain development and disease

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Abstract

Voltage gated sodium channels (VGSCs) were first identified in terms of their role in the upstroke of the action potential. The underlying proteins were later identified as saxitoxin and scorpion toxin receptors consisting of α and β subunits. We now know that VGSCs are heterotrimeric complexes consisting of a single pore forming α subunit joined by two β subunits; a noncovalently linked $\beta 1$ or $\beta 3$ and a covalently linked $\beta 2$ or $\beta 4$ subunit. VGSC α subunits contain all the machinery necessary for channel cell surface expression, ion conduction, voltage sensing, gating, and inactivation, in one central, polytopic, transmembrane protein. VGSC β subunits are more than simple accessories to α subunits. In the more than two decades since the original cloning of $\beta 1$, our knowledge of their roles in physiology and pathophysiology has expanded immensely. VGSC β subunits are multifunctional. They confer unique gating mechanisms, regulate cellular excitability, affect brain development, confer distinct channel pharmacology, and have functions that are independent of the α subunits. The vast array of functions of these proteins stems from their special station in the channelome: being the only known constituents that are cell adhesion and intra/extracellular signaling molecules in addition to being part of channel complexes. This functional trifecta and how it goes awry demonstrates the power outside the pore in ion channel signaling complexes, broadening the term channelopathy beyond defects in ion conduction.

Introduction

Voltage gated sodium channels (VGSCs) were first identified functionally in terms of their role in the upstroke of the action potential (AP) (Hille 2001). The underlying proteins were later identified as saxitoxin and scorpion toxin receptors consisting of α and β subunits (Hartshorne, Messner et al. 1982, Messner and Catterall 1985). We now know that VGSCs are heterotrimeric complexes consisting of a single pore forming α subunit joined by two β subunits; a noncovalently linked $\beta 1$ or $\beta 3$ and a covalently linked $\beta 2$ or $\beta 4$ subunit (Isom and De Jongh 1992, Isom, Ragsdale et al. 1995, Morgan, Stevens et al. 2000, Yu, Westenbroek et al. 2003). Mammalian genomes contain 9 VGSC α subunits, encoded by the

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SCN(1-10)A genes, and 5 β subunits, encoded by the *SCN(1-4)B* genes (Catterall WA and Waxman 2005). VGSC α subunits contain all the machinery necessary for channel cell surface expression, ion conduction, voltage sensing, gating, and inactivation, in one central, polytopic, transmembrane protein (Auld, Goldin et al. 1988). Early electrophysiological results led to the misconception that β subunits are simple accessories to α subunits (Goldin, Snutch et al. 1986). However, in the 25 years since the original cloning of the first β subunit, their role has expanded immensely (Isom and De Jongh 1992). VGSC β subunits confer unique gating mechanisms, regulate cellular excitability, affect brain development, confer distinct channel pharmacology, and have functions that are independent of the α subunits. The vast array of functions of these proteins stems from their special station in the channelome: being the only known constituents that are cell adhesion and intra/extracellular signaling molecules in addition to being part of channel complexes (Isom, De Jongh et al. 1994, Brackenbury and Isom 2011). This functional trifecta and how it goes awry demonstrates the power outside the pore in ion channel signaling complexes, broadening the term channelopathy beyond defects in ion conduction. This review will focus on VGSC β subunits in the nervous system. To read about β subunit function in heart, cancer, and beyond see: (Brackenbury and Isom 2011, Bao and Isom 2014, Martin, Ufodiama et al. 2015, O'Malley and Isom 2015).

Section 1: Structure and Expression of β Subunits

Excluding $\beta 1b$, the VGSC β subunits have type 1 membrane protein topology consisting of an extracellular N-terminal domain, a single transmembrane segment, and a C-terminal tail (Fig. 1) (Isom and De Jongh 1992, Isom, Ragsdale et al. 1995, Kazen-Gillespie, Ragsdale et al. 2000, Morgan, Stevens et al. 2000, Yu, Westenbroek et al. 2003). The array of functions and interactions imparted by each of these domains make β subunits immensely powerful for such small proteins. β subunit functionality in brain is expanded even further if one takes into account their expression profiles in terms of developmental time course, cell type specificity, and subcellular localization. This section will examine the structure and expression of β subunits in the nervous system.

Section 1 Part 1: The β Subunit Immunoglobulin Domain

Each VGSC β subunit features an extracellular N-terminal domain that contains homology to V-type immunoglobulin (Ig) loops common to the Ig superfamily of cell adhesion molecules (CAMs) (Isom and De Jongh 1992, Isom, Ragsdale et al. 1995, Kazen-Gillespie, Ragsdale et al. 2000, Morgan, Stevens et al. 2000, Yu, Westenbroek et al. 2003). In the β subunits, these domains are capable of both interaction with VGSC α subunits and cell adhesion (Brackenbury and Isom 2011). Each Ig domain consists of antiparallel β sheets stabilized by an intrasubunit disulfide bond (McCormick, Isom et al. 1998, Gilchrist, Das et al. 2013, Namadurai, Balasuriya et al. 2014, Das, Gilchrist et al. 2016, Shimizu, Miyazaki et al. 2016). Early on, β subunit structure was modeled based on the crystal structure of the extracellular domain of myelin P0, a protein containing a single V type Ig loop and single transmembrane domain with modest homology to $\beta 1$ and $\beta 3$ (Isom, Ragsdale et al. 1995, McCormick, Isom et al. 1998, Morgan, Stevens et al. 2000). Crystal structures of $\beta 2$, $\beta 3$, and $\beta 4$ were recently solved (Gilchrist, Das et al. 2013, Namadurai, Balasuriya et al. 2014, Das,

Gilchrist et al. 2016). These structures inform and confirm many insights into β subunit Ig domains garnered from previous biochemical work and show that each subunit contains unique structural features. Approximately one-third of the molecular mass of each β subunit is due to N-linked glycosylation moieties in the extracellular domain (Isom and De Jongh 1992, Isom, Ragsdale et al. 1995, McCormick, Isom et al. 1998, Morgan, Stevens et al. 2000, Yu, Westenbroek et al. 2003, Johnson, Montpetit et al. 2004). Sialic acid terminated glycosylation is necessary for modulation of α subunit gating by β 1, suggesting that electrostatic interactions with voltage sensing domains may occur through modification of these residues (Johnson, Montpetit et al. 2004).

VGSC β 1 and β 3 subunits share the highest sequence homology to each other, while β 2 and β 4 share more homology to each other than to either β 1 or β 3 (Isom and De Jongh 1992, Isom, Ragsdale et al. 1995, Morgan, Stevens et al. 2000, Yu, Westenbroek et al. 2003). Studies using chimeric subunits revealed that β 1 modulates the gating of $\text{Na}_v1.2$ via association with the extracellular domain IV S5-S6 region of that α subunit (McCormick, Srinivasan et al. 1999, Qu, Rogers et al. 1999). This may extend to β 3, given its high sequence homology to β 1, although see (Namadurai et al. 2014). The site of association between β 1 and $\text{Nav}1.4$ was localized to the S5-S6 segments connecting domains I and IV of that α subunit, implicating channel specific regions of interaction (Makita, Bennett et al. 1996). β 2 and β 4 bind to VGSC α subunits differently than β 1 and β 3. A key difference between the Ig loops of the β subunits is the presence of an exposed free cysteine residue in β 2 and β 4 that forms a disulfide bond with α subunits (Isom, Ragsdale et al. 1995, Yu, Westenbroek et al. 2003). Mutation of this residue in β 2 or β 4 abolishes α subunit interactions (Chen, Calhoun et al. 2012, Gilchrist, Das et al. 2013). The cysteine residues in β 2 (Cys-26) or β 4 (Cys-58) responsible for covalent association with α were determined to bind to a corresponding cysteine residue in $\text{Na}_v1.2$ located in the domain II S5-S6 loop (Das, Gilchrist et al. 2016). This residue is absent from the tetrodotoxin-resistant (TTX-R) α subunits $\text{Nav}1.5$, $\text{Nav}1.8$ and $\text{Nav}1.9$, although $\text{Nav}1.5$ and β 2 have been shown to associate through disulfide bonds in cardiac tissue and β 2 modifies $\text{Nav}1.5$ currents in heterologous systems and *in vivo* (Malhotra, Chen et al. 2001, Johnson and Bennett 2006, Bao, Willis et al. 2016). β 2 and β 4 differentially affect channel modulation of α subunits by extracellular μ^- , μO^- and $\mu\text{O}\S^-$ conotoxins relative to β 1 and β 3 (Wilson, Zhang et al. 2011, Zhang, Wilson et al. 2013, Gajewiak, Azam et al. 2014, Wilson, Zhang et al. 2015). In the case of $\mu\text{O}\S^-$ conotoxins, association with α subunits occurs through the same cysteine through which these subunits covalently bind to VGSC α subunits. β 1 and β 3 have no influence on $\mu\text{O}\S^-$ conotoxin effects (Gajewiak, Azam et al. 2014). Thus, as expected from early data showing that VGSCs in brain are heterotrimers, these results suggest that the binding sites for β 1 and β 3 vs β 2 and β 4 on α subunits are mutually exclusive.

In addition to channel modulation, β subunits, in particular β 1, participate in homophilic and heterophilic cell adhesion through their extracellular Ig domains. This property allows β subunits to interact with a wide variety of proteins in both *cis* and *trans* configurations, including with identical β subunits in the same membrane or on adjacent cells (Brackenbury and Isom 2011). Importantly, β subunit-mediated cell adhesive interactions can occur both in the presence and absence of VGSC α subunits (Malhotra, Kazen-Gillespie et al. 2000). β subunit-mediated cell adhesion is most well characterized for β 1, which associates

homophilically *in vitro* in *trans*, and heterophilically with the Ig CAMs VGSC $\beta 2$, contactin-1, Nf-186, and NrCam, with the adhesion molecule N-cadherin, and with the extracellular matrix molecule tenascin-R (Isom and Catterall 1996, Malhotra, Kazen-Gillespie et al. 2000, Kazarinova-Noyes, Malhotra et al. 2001, Ratcliffe, Westenbroek et al. 2001, McEwen and Isom 2004). $\beta 2$ associates homophilically as well as heterophilically with $\beta 1$ and tenascin-C, but not with contactin (Srinivasan, Schachner et al. 1998, Xiao, Ragsdale et al. 1999, McEwen and Isom 2004). $\beta 4$ engages in homophilic cell-cell adhesion but its ability to function as a heterophilic CAM has not been tested (Shimizu, Miyazaki et al. 2016). Crystal structure guided photocrosslinking experiments with $\beta 1$ and $\beta 4$ revealed the specific residues responsible for homophilic cell adhesion, further elucidating the structure of VGSC complexes (Shimizu, Miyazaki et al. 2016). Interestingly, $\beta 3$ is predicted to form trimeric complexes that would obscure the analogous residues necessary for *trans* homophilic adhesion (Namadurai, Balasuriya et al. 2014). These results are consistent with earlier data showing that, while $\beta 1$ or $\beta 2$ subunit expression in *Drosophila* S2 cells resulted in the formation of high affinity aggregates, $\beta 3$ expression did not (McEwen, Chen et al. 2009). Overall, however, data on the cell adhesive properties of $\beta 3$ are conflicting, possibly as an effect of differential trimerization based on expression system (Yereddi, Cusdin et al. 2013).

Section 1 Part 2: The Transmembrane and C-terminal Domains of β subunits

β subunits contain a single, α helical transmembrane domain that can confer unique functional properties. For example, the structure of the $\beta 3$ transmembrane domain is proposed to mediate *cis* homophilic interactions allowing for trimerization (Namadurai, Balasuriya et al. 2014). Trimeric $\beta 3$ complexes are capable of forming oligomers with Nav1.5, a departure from the canonical heterotrimeric VGSC structure (Namadurai, Balasuriya et al. 2014). For *Scn1b*, retention of intron 3 through alternative splicing results in the polypeptide $\beta 1b$, a secreted, soluble CAM. In place of a transmembrane domain, $\beta 1b$ contains an alternate C-terminus with an amino acid sequence that is species specific (Kazen-Gillespie, Ragsdale et al. 2000). $\beta 1b$ acts as an extracellular CAM ligand. It is also capable of modulation of $Na_v 1.5$ but not $Na_v 1.1$ or $Na_v 1.3$ in heterologous systems (Watanabe, Koopmann et al. 2008, Patino, Brackenbury et al. 2011). β subunits can also shed their Ig domain following sequential cleavage by β -Site APP-cleaving enzyme 1 (BACE1) and γ -secretase (Wong, Sakurai et al. 2005, Gersbacher, Kim et al. 2010). The resulting soluble extracellular fragment is likely to behave similarly to $\beta 1b$.

β subunit intracellular C-terminal domains are short sequences predicted to have disordered structure (Cusdin, Nietlispach et al. 2010, Lewis and Raman 2011, Namadurai, Balasuriya et al. 2014). Along with the transmembrane segment, the intracellular tails confer additional interactions with VGSC α subunits. Co-expression of $\beta 1$ or $\beta 3$ with Nav1.3 in heterologous cells attenuates lidocaine binding to the channel, suggesting that the β subunit C-terminal domains may occlude the α subunit local anesthetic binding site, located on the S6 helix of domain IV, and accessible only from the intracellular side (Lenkowski, Shah et al. 2003, Catterall 2012). Mutations in *Scn1a* identified an additional α - $\beta 1$ subunit interaction site in the $\beta 1$ C-terminal tail (Spampanato, Kearney et al. 2004). The intracellular domain of $\beta 2$ modulates Nav1.5 gating and the C-terminal tail of $\beta 4$ contains residues capable of intracellular blockade of α subunits to generate resurgent sodium current (I_{Na}) (described in

section 2) (Grieco, Malhotra et al. 2005, Zimmer and Benndorf 2007). β subunit intracellular domains may also impart intracellular regulatory control of α and β subunit activity. For example, phosphorylation of $\beta 3$ residue S161 is necessary for modulation of Nav1.2 I_{Na} (Merrick, Kalmar et al. 2010). The $\beta 1$ intracellular tyrosine residue Y181 is required for $\beta 1$ association with the scaffolding proteins ankyrin-B and ankyrin-G at points of cell-cell contact following *trans* homophilic cell adhesion (Malhotra, Kazen-Gillespie et al. 2000, Merrick, Kalmar et al. 2010). Phosphorylation of this residue prevents ankyrin binding (Malhotra, Koopmann et al. 2002).

Section 1 Part 3: Expression Profile of the β Subunits

The β subunits differ in their expression and posttranslational modifications within different stages of development, in different tissues, between cell types, and subcellular locations. This imparts a specificity that is crucial for normal development and excitability. β subunits are expressed in both excitable and non-excitable cells in brain (O'Malley and Isom 2015). In whole brain, $\beta 1b$ and $\beta 3$ mRNA and protein expression are high during the prenatal and early postnatal period in mammals (Kazen-Gillespie, Ragsdale et al. 2000, Shah, Stevens et al. 2001, Patino, Brackenbury et al. 2011). These subunits then give way to increased expression of $\beta 1$, $\beta 2$, and $\beta 4$ mRNA protein in the first postnatal week and this expression persists through adulthood (Sutkowski and Catterall 1990, Isom and De Jongh 1992, Isom, Ragsdale et al. 1995, Sashihara, Greer et al. 1996, Lein, Hawrylycz et al. 2007). Different cell types in the brain express different β subunits at various levels. *In situ* hybridization and immunohistochemistry show that *Scn1b* and $\beta 1$ expression, respectively, are ubiquitous throughout the brain but with modest heterogeneity (Sashihara, Greer et al. 1996, Blackburn-Munro and Fleetwood-Walker 1999, Lein, Hawrylycz et al. 2007, Brackenbury and Isom 2011, Wimmer, Harty et al. 2015). $\beta 1$ immunohistochemical studies show subcellular localization in similar patterns to that found for α subunits, with high density at axon initial segments (AISs) and nodes of Ranvier in myelinated axons (Chen, Westenbroek et al. 2004, Wimmer, Reid et al. 2010, Kruger, O'Malley et al. 2016). This subcellular localization pattern allows for specialized functions. Exemplary of the importance of subcellular localization is the action of $\beta 1$ at nodes of Ranvier. $\beta 1$ is highly enriched in the nodal gap where it is presumed to modulate VGSC α subunit cell surface expression and gating. However, in the paranodal region $\beta 1$ is engaged in a trimeric axonal-glia cell adhesion complex consisting of axonal $\beta 1$, contactin, and Caspr binding in *trans* to glial neurofascin-155 in the absence of VGSC α subunits (Kazarinova-Noyes, Malhotra et al. 2001, McEwen and Isom 2004). $\beta 1$ expression at the growth cones of developing neurons mediates several important functions where it is proposed to act both as a CAM and as a modulator of I_{Na} (Brackenbury, Calhoun et al. 2010). *Scn1b* is also the mostly highly expressed β subunit gene in the peripheral nervous system (PNS), where it is particularly enriched in medium and large diameter A fibers with lower expression in C fibers (Blackburn-Munro and Fleetwood-Walker 1999, Lopez-Santiago, Pertin et al. 2006, Zhao, O'Leary et al. 2011).

Scn2b expression is similarly ubiquitous in adult brain as assessed through *in situ* hybridization (Blackburn-Munro and Fleetwood-Walker 1999, Lein, Hawrylycz et al. 2007). Similar to $\beta 1$, $\beta 2$ protein is highly expressed at the AIS and nodes of Ranvier. *Scn2b* is also

expressed in the PNS, where its deletion results in loss of responsiveness to inflammatory and neuropathic pain but not mechanosensation, again suggesting cell type specific functions (Lopez-Santiago, Pertin et al. 2006, Zhao, O'Leary et al. 2011).

While *Scn3b* mRNA and protein are expressed ubiquitously throughout the developing CNS, in adult mice expression is greatly attenuated except in specific structures, most highly in hippocampus and others (Morgan, Stevens et al. 2000, Lein, Hawrylycz et al. 2007). This differs in human brain, where *SCN3B* expression remains highly expressed through adulthood similarly to *SCN3A* (Chen, Dale et al. 2000, Lein, Hawrylycz et al. 2007). PNS expression of *Scn3b* is especially high in unmyelinated C fibers and is low in the other fiber types (Lopez-Santiago, Pertin et al. 2006, Zhao, O'Leary et al. 2011).

Scn4b is the most restricted of the β subunits in its expression profile. *Scn4b* mRNA and protein are highly expressed in several neuronal populations, many of which are known for spontaneous or burst firing APs (Lein, Hawrylycz et al. 2007, Lewis and Raman 2014). The highest expression of $\beta 4$ protein and *Scn4b* mRNA are found in the striatum and cerebellar purkinje neurons but are also highly expressed in other restricted brain regions (Lein, Hawrylycz et al. 2007, Miyazaki, Oyama et al. 2014). $\beta 4$ subcellular localization is similar to that of the other β subunits, being enriched at the AIS, nodes of Ranvier, and along the length unmyelinated axons (Buffington and Rasband 2013, Miyazaki, Oyama et al. 2014). In the PNS $\beta 4$ is most highly expressed in medium and large A fibers (Lopez-Santiago, Pertin et al. 2006, Zhao, O'Leary et al. 2011).

While not much is known about the trafficking of β subunits down the axon, this process likely involves interactions with VGSC α subunits. Disruption of the disulfide bond between α subunits and $\beta 2$ or $\beta 4$ impairs the ability of β subunits to traffic to axons and nodes of Ranvier (Chen, Calhoun et al. 2012, Buffington and Rasband 2013). Similarly, an *Scn1b* mutation that interferes with α - $\beta 1$ association results in failure of the mutant $\beta 1$ to traffic from the soma to the AIS and nodes of Ranvier (Kruger, O'Malley et al. 2016). This suggests $\beta 1$ subunit enrichment in axonal domains is dependent on trafficking of α - β complexes rather than to β subunit independent trafficking. This would imply dynamic association of $\beta 1$ with α subunits, as $\beta 1$ in the paranode of nodes of Ranvier is not associated with α subunits (Kazarinova-Noyes, Malhotra et al. 2001, McEwen and Isom 2004). β subunits are also expressed in various glia where they may function as cell adhesion guides and cues for neurodevelopment, including coordinating neurite outgrowth, axonal fasciculation, and neuronal migration (Brackenbury and Isom 2011).

Section 2: β Subunits in Physiology and Development

The cell type specificity of β subunit expression, β subunit specific interactions with other proteins, and unique β subunit structural features outlined in section 1 are important for cell type and brain region specific control of excitability and development. In the following section we will examine the physiological functions of the β subunits at the level of control of VGSC and voltage gated potassium (VGKC) channel currents, structural development in the nervous system, and impacts on excitability.

Section 2 Part 1: β Subunits Alter VGSC Properties

VGSCs undergo a series of conformational changes resulting in several channel states necessary for neuronal AP firing. The β subunits have specific effects on these states and contribute unique gating mechanisms. The “closed” (deactivated) state dominates at negative membrane potentials. In this state, channels do not pass ions. Upon membrane depolarization, the channels undergo conformational shifts to the “open” state, allowing passage of ions. Channels may then revert back to the closed state or undergo the process of “inactivation,” whereby the intracellular face of the channel is blocked by an inactivating “particle” and ion passage is blocked. Relief from inactivation occurs upon hyperpolarization for extended periods of time and is much slower than deactivation, leading to the refractory period between APs (Hille 2001). Early work expressing α subunits in heterologous systems showed that α subunits alone contain all the machinery necessary for these processes (Noda, Ikeda et al. 1986, Tomiko, Rosenberg et al. 1986, Recio-Pinto, Duch et al. 1987). However, the activation and inactivation kinetics of TTX-sensitive α subunits expressed in *Xenopus* oocytes without β subunits are slower than properties observed for native VGSC expression. The voltage dependence of I_{Na} is also altered (Goldin, Snutch et al. 1986, Auld, Goldin et al. 1988, Trimmer, Cooperman et al. 1989, Joho, Moorman et al. 1990). The first indication that β subunits were necessary to reconstitute native channel properties was found by coinjection of low molecular weight brain mRNA (containing β subunit transcripts) with high molecular weight mRNA (containing α subunit transcripts). The low molecular weight mRNA fraction conferred native kinetics and voltage dependence on I_{Na} expressed by the high molecular weight mRNA fraction (Krafte, Snutch et al. 1988). Cloning of *Scn1b* and *Scn2b* cDNAs and their coexpression with α subunits in *Xenopus* oocytes revealed that β subunits are indeed responsible for these differences as well as promotion of the surface expression of α subunits (Isom and De Jongh 1992, Isom, Ragsdale et al. 1995).

The effects of β subunits on channel cell surface expression, activation, and inactivation are dependent on the particular β subunit expressed, the chosen experimental expression system, and which α subunit is coexpressed. An overview of the effects of β subunits on channel gating and trafficking in heterologous cells is provided in (Calhoun and Isom 2014). Some possible reasons for these differences include background expression of other β subunits in some heterologous cell lines and cell line dependent differential posttranscriptional processing of β subunits. Sialic acid terminated glycosylation in particular seems to be important for modulation of α subunit gating by $\beta 1$. Sialic acid terminated glycosylation on residues within the $\beta 1$ Ig loop may confer electrostatic interactions with residues in α subunit voltage sensing domains. In addition, the presence of a high degree of sialic acid terminated glycosylation in $Na_v1.4$ is thought to confer resistance to modulation by or association with $\beta 1$. This is restored by mutating *Scn4a* cDNA to contain non-glycosylatable residues. This modification is also necessary for the effects of β subunits in promoting $Na_v1.4$ cell surface expression, and thus may facilitate intersubunit association rather than mediate gating differences in skeletal muscle (Johnson, Montpetit et al. 2004). Differential sialic acid modification was also found to affect $\beta 2$ modulation of $Nav1.5$ while, in the same study, $Nav1.2$ was modulated regardless of α subunit sialylation status (Johnson and Bennett 2006). The epilepsy-linked $\beta 1$ -C121W mutation in *Scn1b* results in decreased $\beta 1$ subunit

glycosylation and loss of its capacity to modulate VGSC properties and to traffic out of the soma to the axon in mouse neurons (Meadows, Malhotra et al. 2002, Tammaro, Conti et al. 2002, Kruger, O'Malley et al. 2016). This raises the possibility that control of glycosylation of β subunits, especially sialylation, may be important in subcellular targeting and α - β subunit association. Interestingly $\beta 1$ and $\beta 3$ oppositely affect glycosylation of Nav1.7 in heterologous systems, showing β subunits can also control gating of α subunits via regulation of their posttranscriptional processing (Laedermann, Syam et al. 2013). Modulation of I_{Na} by β subunits is also dependent on phosphorylation status. Phosphorylation of S161 in the C-terminal tail of $\beta 3$ is necessary for modulation of Nav1.2 I_{Na} in heterologous cells (Merrick, Kalmar et al. 2010). Phosphomimetic mutation of residue Y181, located in the $\beta 1$ C-terminal tail, results in the loss of $\beta 1$'s capacity to modulate Nav1.2 mediated I_{Na} and surface expression, without interfering with α - β subunit association (McEwen, Meadows et al. 2004). Tyrosine phosphorylation of $\beta 1$ is likely functionally relevant in neurons, as the $\beta 1$ intracellular domain is known to interact with receptor protein tyrosine phosphatase β (RPTP- β) and likely *Fyn* kinase in mouse brain, as *Fyn* null neurons lack $\beta 1$ -mediated neurite outgrowth (Ratcliffe, Qu et al. 2000, Brackenbury, Davis et al. 2008). In contrast to $\beta 1$, $\beta 2$ does not interact with RPTP- β , suggesting β subunit specificity of association with certain phosphorylation enzymes (Ratcliffe, Qu et al. 2000). Thus, differential posttranscriptional modifications to β subunits, including glycosylation and phosphorylation, may lend modularity to channel activity in a cell type and subcellular region specific manner.

Section 2 Part 2: *In Vivo* Effects of β subunits on VGSC Function

Surprisingly, despite high expression throughout the hippocampus and large effects on I_{Na} in heterologous systems, acutely dissociated *Scn1b* null whole hippocampal pyramidal neurons or CA3 bipolar neurons show no changes in I_{Na} density or voltage dependent properties (Chen, Westenbroek et al. 2004, Patino, Claes et al. 2009). This may be due to the process of dissociation, compensation by other β subunit gene expression, or only subsets of neurons being affected by *Scn1b* deletion. The latter is likely, since the mix of several pyramidal neuron populations present in whole hippocampal neuron dissociations could obscure differences in specific hippocampal fields. Notably, in *Scn1b* null CA3 but not CA1 pyramidal neurons or dentate granule neurons, there is a reduction in the expression of $Na_v1.1$ and an increase in the expression of $Na_v1.3$ measured by immunohistochemistry (Chen, Westenbroek et al. 2004). Fittingly, acutely dissociated dentate granule neurons and CA1 pyramidal neurons show no changes in I_{Na} density, kinetics, or voltage dependent properties (Uebachs, Opitz et al. 2010, Uebachs, Albus et al. 2012). In contrast, acutely dissociated *Scn1b* null dorsal root ganglion (DRG) neurons show a depolarizing shift in the voltage dependence of TTX-S I_{Na} inactivation, reduced cell surface expression of $Na_v1.9$, and a prolonged recovery from inactivation of TTX-R I_{Na} compared to wildtype (WT) (Lopez-Santiago, Brackenbury et al. 2011). While cultured cerebellar granule neurons show no change in the amount or voltage dependence of transient I_{Na} , they do have differences in α subunit expression and decreased resurgent I_{Na} , as discussed in more detail in section 2 parts 3 and 4 (Brackenbury, Calhoun et al. 2010).

Scn2b deletion also shows cell type dependent effects in native neurons. Cultured hippocampal neurons from *Scn2b* null mice have reduced surface expression of TTX-S VGSCs. Acutely dissociated *Scn2b* null hippocampal pyramidal neurons, but not dentate granule neurons, have reduced I_{Na} density (Chen, Bharucha et al. 2002, Uebachs, Opitz et al. 2010). *Scn2b* deletion also affects the gating properties of VGSCs, slowing the kinetics of activation and inactivation in dissociated hippocampal neurons. *Scn2b* null small fast DRG neurons show a 50% reduction in transient I_{Na} , reduced $Na_v1.7$ expression, and slowed kinetics of activation and inactivation. These effects of *Scn2b* appear to be specific to TTX-S channels, as TTX-R I_{Na} in the PNS are unchanged, perhaps due to the lack of $\beta 2$ -binding cysteine residues in the TTX-R PNS channels Nav1.8 and Nav1.9, as discussed in section 1 (Lopez-Santiago, Pertin et al. 2006). Differential effects of *Scn2b* deletion on VGSC α subunit subtypes within the same neuron demonstrate that β subunits modulate channels in precise ways *in vivo*.

The effects of $\beta 3$ in the nervous system *in vivo* are not well studied, however *Scn3b* deletion results in reduced transient I_{Na} and hyperpolarized voltage dependence of inactivation in cardiomyocytes, suggesting the effects of $\beta 3$ observed in heterologous system may hold *in vivo* (Hakim, Gurung et al. 2008).

Scn4b knockdown in cultured cerebellar granule neurons results in reduced transient I_{Na} and hyperpolarization of the voltage dependence of transient I_{Na} availability (Bant and Raman 2010). *Scn4b* null mice show no changes in transient I_{Na} in striatum, but see below for effects on special gating schemes (Miyazaki, Oyama et al. 2014). Overexpression of *Scn4b* cDNA in cultured hippocampal CA3 neurons, which do not natively express $\beta 4$, hyperpolarizes and decreases the slope factor of the voltage dependence of availability, suggesting effects of $\beta 4$ in heterologous systems may translate to neurons (Aman, Grieco-Calub et al. 2009).

Section 2 Part 3: Role of β subunits in Resurgent and Persistent Sodium Current

β subunits play roles in special gating schemes that differ from the traditional transient I_{Na} described by Hodgkin and Huxley (Hille 2001). Resurgent I_{Na} is a specialized current in which traditional inactivation is impaired by blockade of the intracellular face of the pore, which is relieved more quickly by hyperpolarization than traditional inactivation. Upon membrane potential repolarization, the channel is able to quickly convert to the open state without the prolonged recovery interval imparted by traditional inactivation. This current activates quickly upon repolarization during an AP and promotes cell type dependent spontaneous, repetitive, or burst firing of APs (Lewis and Raman 2014). The C-terminal tail of $\beta 4$ is the only known endogenous molecule to generate resurgent I_{Na} (Grieco, Malhotra et al. 2005, Lewis and Raman 2014). The residues KKLITFILKKTREK in the C-terminal tail of the mouse $\beta 4$ subunit are required for resurgent I_{Na} in cerebellar purkinje and granule neurons (Grieco, Malhotra et al. 2005). Consistent with this result, *Scn4b* null mice lose resurgent I_{Na} in striatal projection neurons (Miyazaki, Oyama et al. 2014). While $\beta 4$ is the only identified mediator of resurgent I_{Na} to date, there are neuronal populations that express this current but do not express $\beta 4$, suggesting the involvement of other proteins (Lewis and Raman 2014). The ability of $\beta 4$ to generate resurgent I_{Na} is α subunit dependent, with

Nav1.6 carrying proportionally more than the other α subunits (Lewis and Raman 2014). *Scn1b* null cultured granule neurons have reduced resurgent I_{Na} , likely due to decreased expression of this α subunit (Brackenbury, Calhoun et al. 2010).

In addition to reduced resurgent I_{Na} , *Scn4b* null striatal projection neurons also have reduced persistent I_{Na} , defined as the fraction of transient I_{Na} that does not inactivate (Miyazaki, Oyama et al. 2014). Persistent I_{Na} promotes repetitive firing, facilitates the summation of excitatory postsynaptic currents, and contributes to determination of firing threshold (Crill 1996). Knockdown of $\beta 4$ in cultured cerebellar granule neurons reduced both resurgent and persistent I_{Na} (Aman, Grieco-Calub et al. 2009). Inclusion of the C-terminal $\beta 4$ peptide restored resurgent I_{Na} in these neurons and induced it in neurons that do not typically have resurgent I_{Na} (Grieco, Malhotra et al. 2005). Interestingly, in contrast to the $\beta 4$ C-terminal peptide, expressing the full-length $\beta 4$ protein does not generate resurgent I_{Na} in heterologous systems. It does, however, destabilize inactivation; tripling persistent I_{Na} amplitude (Aman, Grieco-Calub et al. 2009). $\beta 4$ is not unique in its ability to impact persistent I_{Na} . $\beta 3$, but not $\beta 1$ or $\beta 2$, when coexpressed in heterologous systems with Nav1.2, increases persistent I_{Na} (Qu, Curtis et al. 2001). *Scn1b* has effects on both persistent and resurgent I_{Na} *in vitro* and *in vivo*. *Scn1b* null CGNs have reduced resurgent I_{Na} and *Scn1b* null DRGs have reduced persistent TTX-R I_{Na} (Brackenbury, Calhoun et al. 2010, Lopez-Santiago, Brackenbury et al. 2011). This effect in CGNs may be due more to the loss of effects of *Scn1b* on Nav1.6 subunit expression, which is known to disproportionality carry resurgent and persistent I_{Na} relative to other α subunits (Lewis and Raman 2014).

In summary, many neurons undergo dynamic changes in their firing profile, which may be controlled by the levels of persistent and resurgent I_{Na} expressed (Krahe and Gabbiani 2004, Paul, DeWoskin et al. 2016). β subunits may play critical roles in neuronal firing based on their impacts to these currents, and thus impart dynamic control over neuronal firing properties.

Section 2 Part 4. β Subunits Affect Trafficking and Expression of VGSC α subunits *In Vivo*

The early heterologous expression studies of $\beta 1$ and $\beta 2$ revealed that, in addition to modulation of gating, these subunits also impact channel surface expression, largely through effects on trafficking and cell surface half-life of channels rather than through their transcriptional control (but see below) (Schmidt, Rossie et al. 1985, Schmidt and Catterall 1986, Isom and De Jongh 1992, Isom, Ragsdale et al. 1995). Association with $\beta 2$ is the final step in VGSC biosynthesis, promoting α subunit insertion into the plasma membrane (Schmidt, Rossie et al. 1985, Schmidt and Catterall 1986). In contrast, “free” α subunits not associated with a disulfide-linked β subunit are located inside the cell and are inactive (Schmidt, Rossie et al. 1985). In addition, α subunits covalently associated with β subunits are removed from the cell surface more slowly than free α subunits (Schmidt and Catterall 1986). Consistent with these data, hippocampal neuron cultures from *Scn2b* null mice show a reduction in α subunits expressed at the cell surface (Chen, Bharucha et al. 2002). Interestingly, the function of $\beta 2$ on VGSC surface expression in mammalian cells appears to be dependent on $\beta 1$, and this point is discussed in more detail in the next section. In contrast to $\beta 2$, $\beta 4$ does not appear to contribute to surface expression of α subunits; *Scn4b* null

neurons show no apparent reductions in transient sodium current (Miyazaki, Oyama et al. 2014).

Scn1b impacts α subunit expression and trafficking in an isoform specific manner *in vivo*. In CGNs, *Scn1b* deletion in mice causes a reduction in Nav1.6 and an increase in Nav1.1 expression at the CGN AIS, leaving total current density unchanged (Brackenbury, Calhoun et al. 2010). In rat brain, while “free” α subunits not disulfide linked to β subunits account for 21% of VGSCs in the soma, they are wholly undetectable in synaptosomes. This suggests that $\beta 2$ or $\beta 4$ subunits may play roles in directing α subunits to the synapse (Schmidt, Rossie et al. 1985, Schmidt and Catterall 1986). More recent work shows that $\beta 3$ mediates clustering of $\text{Na}_v 1.5$ channels in heterologous systems, possibly through *cis* homophilic interactions resulting in $\beta 3$ trimer formation (Namadurai, Balasuriya et al. 2014). VGSC α subunit single molecule tracking microscopy in fetal rat hippocampal neuron cultures showed that VGSCs exist in clustered microdomains (Akin, Solé et al. 2016). While the role of β subunits in this clustering is unknown, data showing the effects of $\beta 3$ on VGSC clustering in culture may lend important insights into this process, since $\beta 3$ is highly expressed in fetal and postnatal rodent brain. $\beta 3$ also may affect α subunit expression through masking of an endoplasmic reticulum retention/retrieval motif (Zhang, Li et al. 2008). *Scn4b* deletion does not affect axonal expression of Nav1.2 in striatal projection neurons, suggesting that it may not play roles in channel trafficking but instead its function may be limited to specialized impacts on persistent and resurgent sodium currents (Miyazaki, Oyama et al. 2014).

β subunits can affect VGSC α subunit transcription or translation. In *Scn1b* null hippocampus, CA3, but not CA1 or dentate gyrus, pyramidal neurons have reduced Nav1.1 and increased Nav1.3 expression (Chen, Westenbroek et al. 2004). In addition, Nav1.9 protein is reduced in *Scn1b* null DRGs (Lopez-Santiago, Brackenbury et al. 2011). $\beta 2$ is also able to affect expression of specific α subunits. In DRGS, mRNA for Nav1.7, Nav1.6 and Nav1.3 is reduced, while Nav1.1 and Nav1.7 protein are reduced, with no change in Nav1.6 (Lopez-Santiago, Pertin et al. 2006). Many of these effects on specific VGSC α subunit expression may be due to transcriptional regulatory effects of β subunits, as discussed in the next section.

Section 2 Part 5. Proteolytic Processing of β subunits

VGSC β subunits are targets for proteolytic cleavage, with the resulting products demonstrating specific autonomous functions. The β subunits contain cleavage sites for sequential cleavage by BACE1 and γ -secretase (Wong, Sakurai et al. 2005, Gersbacher, Kim et al. 2010). $\beta 2$, and likely the other β subunits, contains an additional cleavage site for α -secretase enzyme ADAM10 in the extracellular portion (Kim, Ingano et al. 2005). BACE1 cleavage results in shedding of the extracellular β subunit domain. For $\beta 1$, this cleaved polypeptide fragment is thought to be capable of functioning as a ligand for cell adhesion, similar to $\beta 1b$ (Kim, Ingano et al. 2005, Wong, Sakurai et al. 2005). Interestingly, while expression of full-length $\beta 4$ subunits with α subunits in heterologous systems does not recapitulate resurgent I_{Na} , cleavage of $\beta 4$ by BACE1 slows the decay of resurgent I_{Na} , suggesting that proteolytic processing of $\beta 4$ may be involved in generation of resurgent I_{Na} .

(Huth, Rittger et al. 2011). Because the $\beta 4$ residues responsible for generating resurgent I_{Na} are located within the C-terminal tail, sequential cleavage by γ -secretase, following the action of BACE1, may also be important in this mechanism. Formation and nuclear translocation of the γ -secretase cleaved $\beta 2$ C-terminal fragment has been shown to lead to increased Nav1.1 subunit mRNA and protein expression in neurons, suggesting a role for β subunits in the regulation of α subunit transcription (Kim, Carey et al. 2007). While similar roles for the other β subunits have not been investigated, their processing by this cleavage pathway and effects on specific α subunit expression may suggest similar mechanisms. Thus, β subunits may also affect neuronal firing through the modulation of channel transcription.

Section 2 Part 6. β Subunit Crosstalk

VGSC β subunits can also impact the actions of each other. Coexpression of $\beta 1$ and $\beta 2$ in oocytes with Nav1.2 increases I_{Na} density greater than either β subunit alone, suggesting synergistic action (Isom, Ragsdale et al. 1995). In Chinese hamster lung fibroblasts, $\beta 2$ expression does not increase the surface expression of Nav1.2 unless $\beta 1$ is also expressed (Kazarinova-Noyes, Malhotra et al. 2001, McEwen, Meadows et al. 2004). This seems to be the case for endogenous expression as well; mice that are homozygous for both the *Scn1b* and *Scn2b* null alleles have reduced I_{Na} density in cerebellar purkinje neurons but I_{Na} density is not different in mice null for *Scn1b* or *Scn2b* alone (Grieco, Malhotra et al. 2005). This result suggests there is a capacity for $\beta 1$ and $\beta 2$ to compensate for each other *in vivo*, in spite of evidence that these subunits bind to different locations on α subunits. This may be the case for other subunits as well. *Scn3b* null mice have apparently normal nervous system development, suggesting compensatory *Scn1b* expression (Hakim, Gurung et al. 2008, Hakim, Brice et al. 2010). Coexpression of $\beta 1$ with $\beta 4$ overcomes $\beta 4$ induced persistent I_{Na} in heterologous cells and neurons. Additionally, the $\beta 4$ mediated hyperpolarized activation and inactivation of Nav1.1 I_{Na} are antagonized by $\beta 1$. In contrast, $\beta 1$ functions appear to be relatively unaffected by $\beta 4$ co-expression, as cells expressing both subunits match the electrophysiological profile of $\beta 1$ expression alone. These data suggest opposing roles of $\beta 1$ and $\beta 4$ in the same trimeric channel complex, with $\beta 4$ acting as an excitation accelerator and $\beta 1$ acting as an overriding brake (Aman, Grieco-Calub et al. 2009). While the binding sites on α subunits for $\beta 1$ and $\beta 3$ are predicted to be competitive, there is evidence that $\beta 1$ and $\beta 3$ coexpression can generate properties not present with either alone when coexpressed with Nav1.5 (Ko, Lenkowski et al. 2005). This may be specific to the α subunit, as similar experiments with Nav1.8 show the opposing shifts in channel availability with $\beta 1$ and $\beta 3$ seem to cancel when coexpressed in the same oocyte. In the same study, $\beta 1$ and $\beta 2$ coexpression revealed a depolarizing voltage shift in availability, contrary to the hyperpolarizing shift of $\beta 1$ alone or the absence of effect of $\beta 2$ alone (Vijayaragavan, Powell et al. 2004). β subunits can also affect expression of other β subunits *in vivo*. Quantification of β subunit proteins in *Scn4b* null mice showed increased expression of $\beta 1$ and reduced $\beta 2$ in spinal cord and cerebellum, with no effect on their expression levels in striatum or cortex (Miyazaki, Oyama et al. 2014). In DRG neurons, *Scn2b* null mice have decreased expression of *Scn3b* and *Scn4b* but not *Scn1b* mRNA (Lopez-Santiago, Pertin et al. 2006). This cell type specific regulation of β subunit mRNA expression by other β subunits further highlights the precise control of β subunit function *in vivo*.

Section 2 Part 7: β Subunits Modulate Potassium Channels

β subunits also affect some potassium currents (I_K), creating the potential for cross-talk between VGKCs and VGSCs. $\beta 1$ subunits interact with $K_v4.2$ and $K_v4.3$, major contributors to A-type I_K in brain. This interaction was first uncovered in heterologous expression studies (Deschênes and Tomaselli 2002). Later, an unbiased proteomic approach designed to discover novel $K_v4.2$ interacting partners in rodent brain identified VGSC $\beta 1$. In the same study, investigators found that heterologous co-expression of $\beta 1$ with $K_v4.2$ increases I_K density. In addition, shRNA knockdown of $\beta 1$ in cultured cortical neurons decreased A-type I_K (Marionneau, Carrasquillo et al. 2012). $\beta 1$ also modulates several other VGKCs when co-expressed in *Xenopus* oocytes, including $K_v4.3$, $K_v1.1$, $K_v1.3$, $K_v1.6$, and $K_v7.2$. $\beta 1$ did not, however, modulate $K_v3.1$ in this study, suggesting that interactions are moderately specific to channel type (Nguyen, Miyazaki et al. 2012). It will be important to test these interactions in native neurons and cardiomyocytes because $\beta 1$ interactions are known to be dependent on cell type and these interactions may be limited to heterologous overexpression systems. VGKC modulation by $\beta 1$ may be cell type dependent as well. CA1 pyramidal neurons express a high level of $K_v4.2$ mediated A-type I_K but their excitability is largely unaffected in *Scn1b* null or homozygous mutant neurons (Chen, Yuan et al. 2006, Patino, Claes et al. 2009, Uebachs, Opitz et al. 2010, Reid, Leaw et al. 2014). *Scn1b* null mice exhibit reduced transient outward I_K in DRG neurons, demonstrating that the effects of $\beta 1$ on VGKCs is not limited to the brain (Lopez-Santiago, Brackenbury et al. 2011). Potential effects of other VGSC β subunits on VGKCs in the nervous system have not been investigated, however, large effects on I_K in *Scn2b* null mouse cardiomyocytes suggest that other β subunits may also modulate VGKC function (Bao, Willis et al. 2016).

Section 2 part 8: β Subunit Function in Brain Development

VGSC β subunits function in nervous system development on several levels, including neurite outgrowth, axonal pathfinding, dendritic arborization, neuronal patterning, and cell migration. Neurite extension of WT CGNs plated on $\beta 1$ expressing fibroblast monolayers is increased compared to control monolayers and requires $\beta 1$ - $\beta 1$ *trans* homophilic cell adhesion. In contrast, $\beta 2$ expressing fibroblast monolayers reduce neurite outgrowth of WT CGNs and $\beta 4$ has no detectable effects. $\beta 1$ function in neurite outgrowth requires both cell-cell adhesion and sodium channel activity, particularly current carried by $Na_v1.6$. Deletion of *Cntn1*, *Fyn*, or *Scn8a* or the addition of TTX abrogated $\beta 1$ -mediated neurite outgrowth in CGNs. Unlike other CAMs, $\beta 1$ dependent neurite outgrowth is independent of the FGF mediated neurite outgrowth pathway. *Scn1b* null mice have several nervous system structural defects that may be directly related to altered neurite outgrowth. These include cerebellar parallel fiber defasciculation, CGN axonal mistargeting, dentate granule neuron axonal mistargeting, corticospinal tract defasciculation, and decreased dendritic arborization in pyramidal neurons of the subiculum (Brackenbury, Davis et al. 2008, Brackenbury, Calhoun et al. 2010, Brackenbury, Yuan et al. 2013, Reid, Leaw et al. 2014). Structural defects in *Scn1b* null brain go beyond the regulation of neuronal process extension, also impacting cell migration, proliferation, and neuronal patterning defects. In the cerebellum there is an expansion of the external germinal layer and in the hippocampus there is increased proliferation of dentate granule neurons, ectopic Prox1 positive neurons, and increased

thickness of the dentate gyrus granule cell layer with a reduction in dentate granule neuron number (Brackenbury, Davis et al. 2008, Brackenbury, Yuan et al. 2013).

Section 2 part 9: Differential Effects of β Subunits on Excitability

The wide array of $\beta 1$ -mediated effects on neuronal excitability is consistent with the multi-functionality of $\beta 1$. Several of these effects can be linked directly to modulation of VGSC α subunit expression and function, while others are attributable to VGKC modulation or altered neuronal morphology. *Scn1b* deletion in mice results in differential effects on excitability at the single neuron level with some populations showing increased excitability, some decreased, and others unaffected. Cultured *Scn1b* null CGNs show a decrease in the number of APs fired in response to depolarizing current injections compared to WT (Brackenbury, Calhoun et al. 2010). There are no measureable changes in the level of transient I_{Na} in these cells, but instead a decrease in the level of resurgent I_{Na} , which is known to be important in repetitive firing of these neurons (Brackenbury, Calhoun et al. 2010, Lewis and Raman 2014). Many *Scn1b* null neuron populations, including dentate granule neurons, CA1 pyramidal neurons, and hippocampal interneurons, have unaffected excitability profiles compared to WT (Patino, Claes et al. 2009, Uebachs, Opitz et al. 2010, Uebachs, Albus et al. 2012). *Scn1b* null optic nerve has reduced speed of the fastest component of the compound AP, suggesting decreased excitability (Chen, Westenbroek et al. 2004).

The reported differential effects on excitability appear to be dependent on different functions of *Scn1b*. *Scn1b* null cortical layer 5 pyramidal neurons fire more APs compared to WT with an increased action potential half width, indicating loss of $\beta 1$ modulation of $K_{v4.2}$ may be a factor (Marionneau, Carrasquillo et al. 2012). However, in homozygous knockin mice expressing the epilepsy causing *Scn1b*-C121W mutation, this population is unaffected (Reid, Leaw et al. 2014). While these results are seemingly at odds, they may provide clues to even more nuanced differences in the effects of *Scn1b* on neuronal excitability. The $\beta 1$ -C121W mutation interferes with both α subunit interaction and cell adhesion but with unknown impact on modulation of VGKCs (Meadows, Malhotra et al. 2002, Kruger, O'Malley et al. 2016). This may indicate subpopulations of the same neuron type in different regions of cortex may be affected in different ways or that C121W mutants may modulate excitability differently than total loss of *Scn1b*. The latter may be the case because C121W heterozygous mice have increased seizure susceptibility relative to *Scn1b*^{+/-} mice (Kruger, O'Malley et al. 2016). Hyperexcitability has been found in layer 2/3 pyramidal neurons and pyramidal neurons of the subiculum in mice homozygous for the $\beta 1$ -C121W mutation. These increases in excitability can be attributed to differences in structural development of these neurons, as they have increased input resistance and reduced cell surface area, measured by whole cell capacitance and immunohistochemistry, respectively (Reid, Leaw et al. 2014). Interestingly, this may be a way that reductions in VGSC function can drive hyperexcitability via cross-talk between I_{Na} and neuronal development.

The effects of *Scn2b* on excitability are not as well studied as those of *Scn1b*. We do know, however, that *Scn2b* null mice have increased threshold and reduction in amplitude of extracellularly recorded compound APs in the optic nerve, suggesting that reduced levels of

VGSCs at the cell surface result in reduced excitability. *Scn2b* deletion also increases susceptibility to pilocarpine-induced seizures, suggesting aberrant neuronal excitability elsewhere in the CNS (Chen, Bharucha et al. 2002). While the effects of *Scn2b* deletion on DRG AP firing have not been measured, altered VGSC function and reduced pain sensitivity in *Scn2b* null mice suggest reduced DRG neuron excitability (Lopez-Santiago, Pertin et al. 2006).

The effects of *Scn3b* on nervous system excitability have not been reported, but there is extensive work showing impacts on cardiomyocyte excitability (Hakim, Gurung et al. 2008, Hakim, Brice et al. 2010).

Scn4b's effects on neuronal excitability appear to be largely related to its role in regulating persistent and resurgent I_{Na} . *Scn4b* null mice have reduced repetitive firing of APs in striatal projection neurons and *Scn4b* knockdown in cultured CGNs causes reduced excitability, suggesting that reductions in resurgent I_{Na} impact excitability (Bant and Raman 2010, Miyazaki, Oyama et al. 2014). In a similar manner, reduced persistent and resurgent current in *Scn1b* null CGNs decrease excitability (Brackenbury, Calhoun et al. 2010).

Section 3: β Subunits in Diseases of the Nervous System

β subunits play important roles in both the mechanism and treatment of several disorders of the nervous system. The underlying links between β subunit gene mutations and these disparate diseases result from the numerous biochemical and physiological functions outlined in sections 1 and 2. Some can be linked to effects on neuronal and network excitability, while others may be associated with the effects of β subunits in brain development. The most direct link to disease is between mutations in *SCN1B* and epilepsy. *SCN1B* mutations in human patients are linked to two pediatric epilepsies: generalized epilepsy with febrile seizures plus (GEFS+) and the pediatric epileptic encephalopathy Dravet syndrome (DS). Links also exist between the β subunits and several other disorders including neuropathic pain, neurodegenerative diseases, neurodevelopmental disorders, and sudden infant death syndrome (SIDS). Thus, β subunits are important and novel therapeutic targets due to their unique expression profiles, functions, and pharmacology.

Section 3 Part 1: β Subunits and Epilepsy

Epilepsy is a condition characterized by unprovoked recurrent seizures (Fisher, Acevedo et al. 2014). Up to 40% of epilepsy cases have unknown origin (Beck papers for citation). Exceptions to this are the genetic epilepsies, which can often be linked directly to specific ion channel gene mutations. Mutations in several VGSC genes, including *SCN1B*, are known to cause epilepsy (Meisler, O'Brien et al. 2010). One of the first identified mutations linked to human epilepsy was *SCN1B*-C121W that results in GEFS+ (Wallace, Wang et al. 1998). GEFS+ is characterized by early pediatric seizures associated with fever followed by febrile and afebrile seizures. GEFS+ is one of a multitude of genetic pediatric epilepsies associated with VGSC mutations. The most extreme of these VGSC related epilepsies is Dravet syndrome, a debilitating epileptic encephalopathy resulting in severe epilepsy, ataxia, cognitive impairment, autism, sleep abnormalities, and a high risk of sudden unexpected death in epilepsy (SUDEP) (Scheffer, Zhang et al. 2010, Skluzacek, Watts et al. 2011). More

than 70% of DS cases are caused by *de novo* loss-of-function mutations in *SCN1A*, however a small subset has been identified to have inherited homozygous mutations in *SCN1B* (Patino, Claes et al. 2009, Scheffer, Zhang et al. 2010, Ogiwara, Nakayama et al. 2012, Ramadan, Patel et al. 2017). The first of the *SCN1B* associated DS cases identified was a loss of function mutation, *SCN1B*-R125C that results in failure of $\beta 1$ subunits to traffic to the cell surface (Patino, Claes et al. 2009). Simple loss-of-function is a viable explanation for the mechanism of *SCN1B*-linked DS, as *Scn1b* null mice recapitulate the full DS phenotype, including frequent spontaneous seizures, ataxia, developmental delay, and early lethality (Chen, Westenbroek et al. 2004). This phenotype is more severe than is found in *Scn1a* DS mice. 100% of *Scn1b* null mice die by postnatal day (P)21, which may explain the limited detection of patients with homozygous *SCN1B* mutations in the human population (Chen, Westenbroek et al. 2004). Even though $\beta 1$ -C121W polypeptides are expressed at the neuronal cell surface, mice homozygous for the GEFS+ mutation, *Scn1b*-C121W, recapitulate the phenotype of *Scn1b* null mice, suggesting loss-of-function of a cell surface expressed subunit (Reid, Leaw et al. 2014, Kruger, O'Malley et al. 2016). Interestingly, mice heterozygous for the *Scn1b*-C121W allele have increased susceptibility to febrile seizures compared to mice heterozygous for the *Scn1b* null allele (Kruger, O'Malley et al. 2016). Differences in excitability at the single neuron level are not always consistent between the *Scn1b* null and *Scn1b*-C121W homozygous mouse models (Marionneau, Carrasquillo et al. 2012, Reid, Leaw et al. 2014). Several defects in AP waveforms in *Scn1b*-C121W heterozygous mice are absent in *Scn1b*-C121W homozygous mice (Wimmer, Reid et al. 2010, Reid, Leaw et al. 2014). Thus, *SCN1B* associated GEFS+ and DS likely have distinct mechanisms.

A *SCN1B* mutation specific to $\beta 1b$, G25R, was identified in multiple pedigrees with idiopathic epilepsy (Patino, Brackenbury et al. 2011). Excluding this mutation, all the *SCN1B* epilepsy-causing mutations are within or near the Ig domain, which is important in both α subunit modulation and cell adhesion as described in section 1. Importantly, in the *Scn1b* null mouse, developmental defects appear prior to the onset of seizures or evidence of altered neuronal excitability, suggesting a causative effect of aberrant brain development on altered excitability (Brackenbury, Yuan et al. 2013). As described in section two, the hyperexcitability of homozygous *Scn1b*-C121W subicular pyramidal neurons and cortical layer 2/3 pyramidal neurons appears to be a result of decreased cell size (Reid, Leaw et al. 2014). Importantly, specific deletion of *Scn1a* in forebrain GABAergic interneurons is sufficient to phenocopy DS in mice (Cheah, Frank et al. 2012, Ogiwara, Iwasato et al. 2013). Little is known about altered interneuron function in *Scn1b* models of DS, but initial experiments in hippocampal interneurons suggest that they are not affected (Patino, Claes et al. 2009, Reid, Leaw et al. 2014). Given the brain region and cell type specificity of $\beta 1$ subunit expression discussed in section 2, it is important to expand our understanding of $\beta 1$ function in the CNS to different neuron types. This may be a useful guide for understanding the exact microcircuit components that are altered in DS and GEFS+ and help us to understand how similar epileptic phenotypes may arise from different mechanisms in specific cell populations.

β subunits are also important in the treatment and pathophysiology of other epilepsies. Approximately 30% of epilepsy patients lack adequate seizure control with existing

antiepileptic drugs (AEDs) for unknown reasons (Kwan and Brodie 2000). The actions of β subunits affect the efficacy of two known AEDs, carbamazepine and phenytoin (Lucas, Meadows et al. 2005, Uebachs, Opitz et al. 2010, Uebachs, Albus et al. 2012). A GEFS+-linked *SCN1B* mutation causes a reduction in the efficacy of phenytoin, attenuating the drug's effects on frequency dependent block of sodium current (Lucas, Meadows et al. 2005). The effects of carbamazepine are also affected by β subunits. *Scn1b* null neurons show a paradoxical increase in persistent current when treated with carbamazepine, abolishing the usual spiking attenuation caused by this drug (Uebachs, Opitz et al. 2010, Uebachs, Albus et al. 2012).

VGSC β subunit expression is differentially impacted in several forms of epilepsy. In pilocarpine induced seizures, there is a downregulation of *Scn1b* and *Scn2b* mRNA following status epilepticus with impacts on sodium current consistent with known functions of these two subunits (Gastaldi, Robaglia-Schlupp et al. 1998, Ellerkmann, Remy et al. 2003). This result may be model or species dependent, as there is no evidence for similar increases in temporal lobe epilepsy patients (Gassen, Koen et al. 2008). There is however, a reduction in *SCN3B* and $\beta 3$ expression in the hippocampus of temporal lobe epilepsy patients and increased *SCN1B* and $\beta 1$ in reactive astrocytes of both epileptic and non-epileptic brain trauma patients. (Gorter, Van Vliet et al. 2002, Aronica, Troost et al. 2003, Gassen, Koen et al. 2008, Das, Wallace et al. 2012).

Section 3 Part 2: β Subunits in Sudden Unexpected Death in Epilepsy and Sudden Infant Death Syndrome

Sudden Unexpected Death in Epilepsy (SUDEP) is defined as death in patients with epilepsy that is not due to status epilepticus, trauma or drowning (Nashef, So et al. 2012). SUDEP occurs at a high rate among DS patients, with a 6-fold increase in risk above other epilepsies and 24-fold higher risk of sudden death than in the general population (Skluzacek, Watts et al. 2011). As mentioned above, 100% of *Scn1b*-DS mice die by P21. This phenotype is more severe than for *Scn1a* DS mouse models and other models of SUDEP, such as the *Kcna1* null mouse, which exhibit death rates of ~50% and ~25%, respectively (Chen, Westenbroek et al. 2004, Frank, Mantegazza et al. 2006, Glasscock, Yoo et al. 2010). This positions the *Scn1b* null mouse as a reliable model in the study of SUDEP mechanisms. While the mechanism of SUDEP is not understood, it likely involves a “perfect storm” of catastrophic events. Proposed mechanisms of SUDEP include the effects of seizures on the brain and other organ systems resulting in spreading depression to the brain stem, autonomic dysfunction, cardiac arrhythmias, apnea, or pulmonary edema. SUDEP in DS patients typically occurs in the night and thus may involve defects in the autonomic nervous system, resulting in shutdown of breathing or arousal centers, as well as cardiac arrhythmias (Goldman, Glasscock et al. 2009). In addition to severe seizures, *Scn1b* null mice have cardiac arrhythmias, the combination of which may result in sudden death (Lin, O'malley et al. 2015). SIDS may also be related to β subunit dysfunction. The cause of SIDS remains unclear and likely varies. Sleep position is a known SIDS risk factor but genetic factors including β subunit gene mutations are now known to contribute (Krous, Beckwith et al. 2004, Tan, Pundi et al. 2010). Several instances of SIDS have been linked to *SCN3B* and *SCN4B* mutations (Tan, Pundi et al. 2010). *Scn4b* null mice have spontaneous unexplained

death without an apparent seizure phenotype, but this occurs post development and may be unrelated to SIDS (Miyazaki, Oyama et al. 2014). Mutations in β subunits may be related to SIDS through malfunctions of their roles in the heart, but there are also several overlaps between CNS developmental malformations and reactive astrogliosis in SIDS and the deficits seen in β subunit null models (Kinney, Brody et al. 1991, Cruz-Sánchez, Lucena et al. 1997, Gorter, Van Vliet et al. 2002, Aronica, Troost et al. 2003, Paine, Jacques et al. 2014).

Section 3 Part 3: β subunits and Pain

β subunits are involved in both nociceptive and neuropathic pain, and are expressed in fiber types responsible for pain sensation, as outlined in section 2. Thus, the normal pain sensing pathways that encode information through firing rates may be dependent on the differential expression of β subunits. Deletion of *Scn1b* results in DRG neuron hyperexcitability, predicting that these mice have allodynia (Lopez-Santiago, Brackenbury et al. 2011). In contrast, *Scn2b* deletion results in insensitivity to inflammatory and neuropathic pain paradigms (Lopez-Santiago, Pertin et al. 2006). Several pain models show perturbations of β subunit protein or mRNA expression. *Scn1b* mRNA is increased in dorsal horn neurons in a constrictive nerve injury model (Blackburn-Munro and Fleetwood-Walker 1999). In several injury models the expression levels of $\beta 2$ mRNA and protein are perturbed in a cell type and injury specific manner, suggesting unique pathways for $\beta 2$ regulation in different cell types and injury paradigms (Coward, Jowett et al. 2001, Pertin, Ji et al. 2005, Lopez-Santiago, Pertin et al. 2006). *Scn3b* is upregulated in a fiber type dependent manner in several different nerve injury models, further suggesting that the cell type specificity of β subunit expression is important in the generation of acute and chronic pain (Shah, Stevens et al. 2000, Takahashi, Kikuchi et al. 2003). *Scn4b* siRNA delivered to the DRG interferes with mechanical hypersensitivity in a radicular pain model. In this model, *Scn4b* siRNA interferes with increases in transient and resurgent current present in cultured DRG neurons following inflammation induction (Xie, Tan et al. 2016). $\beta 4$ may be mechanistically important in *SCN9A*-linked paroxysmal extreme pain disorder. In this model, there is an increase in DRG excitability and resurgent current sodium current carried by $\text{Na}_v1.7$, which may be regulated *in vivo* by $\beta 4$ (Theile, Jarecki et al. 2011). The unique expression profile of each β subunit may make them valuable targets for pain therapeutics. For example, because $\beta 1$ subunits are already known to impact the pharmacology of pain related drugs such as lidocaine and several neurotoxins, they may be readily targetable.

Section 3 Part 4: β Subunits in Demyelinating and Neurodegenerative Disorders

There are several lines of evidence to show that β subunits are important in demyelinating and neurodegenerative disorders. *Scn1b* null mice have defects in myelination in the CNS and PNS, including a reduction in the number of nodes of Ranvier in the optic nerve, improper paranodal development in CNS and PNS, brain and spinal cord dysmyelination, and increased axonal degeneration (Chen, Westenbroek et al. 2004). While the axo-glial cell adhesive complexes at paranodes involving $\beta 1$, contactin-1, Caspr, and NF-155 are obvious candidates for this defect, the impact of sodium current defects may also be important, as channel activity is involved in the establishment of myelination (Gibson, Purger et al. 2014). While *Scn2b* null mice have normal myelination, *Scn2b* deletion ameliorates disease

severity in a mouse model of experimental allergic encephalomyelitis (EAE), showing decreased axonal degeneration, fewer demyelinated and dysmyelinated fibers, reduced death, and reduced symptom severity compared to WT mice (Chen, Bharucha et al. 2002, O'Malley, Shreiner et al. 2009). Interestingly, MS patient cerebrospinal fluid has decreased BACE1 activity, suggesting that cleavage of the β subunits may be impacted and thus clinically relevant, as disease severity is correlated with the degree of BACE1 reduction (Mattsson, Axelsson et al. 2009).

Sequential BACE1 and γ -secretase cleavage of β subunits may also be important in Alzheimer's disease. In Alzheimer's disease, cleavage of amyloid precursor protein releases Amyloid β peptide, which accumulates in the brain and forms aggregates (Evin, Barakat et al. 2010). Cleavage of the VGSC β subunits involves this same pathway and may underlie the hyperexcitability and increased seizure risk observed in Alzheimer's (Amatniek, Hauser et al. 2006). β subunits are also indicated in the pathophysiology of several other neurodegenerative diseases. In an amyotrophic lateral sclerosis model, there is downregulation of *Scn1b* and upregulation of *Scn3b* in the ventral dorsal horn, potentially accounting for observed neuronal hyperexcitability there (Nutini, Spalloni et al. 2011). *Scn4b* may be important in Parkinson's disease and Huntington's disease, potentially related to its high expression in neurons in the substantia nigra and striatum (Lein, Hawrylycz et al. 2007, Miyazaki, Oyama et al. 2014). In a model of Parkinson's disease, *Scn4b* mRNA expression, $\beta 4$ protein expression, and the extent of $\beta 4$ glycosylation are increased (Zhou, Zhang et al. 2012). In a model of Huntington's disease, $\beta 4$ expression is reduced in neurons expressing mutant huntingtin (Oyama, Miyazaki et al. 2006). *Scn4b* null mice have decreased resurgent sodium current and hypoexcitability in the striatal projection neurons, representing a possible role of altered excitability in striatal neurodegeneration in Huntington's disease (Miyazaki, Oyama et al. 2014).

Section 3 Part 5: β Subunits in Neurodevelopmental and Neuropsychiatric Disorders

Many epilepsy patients have comorbidities including autism, intellectual disability, impaired sleep, and mood disorders (Steffenburg, Gillberg et al. 1996, Levisohn 2007, Louis 2011, Lau, Ettinger et al. 2012). DS in particular is characterized as having high incidences of autism and intellectual disability and *Scn1a*^{+/-} mice model autism spectrum disease (Han, Tai et al. 2012, Villeneuve, Laguitton et al. 2014). It is unclear if intellectual disability in DS is the result of epilepsy, single neuron excitability, or altered neuronal development. Altered CNS development in *Scn1b* null mice may predict intellectual disability and precede the onset of epilepsy and neuronal hyperexcitability (Brackenbury, Yuan et al. 2013). Mood disorders are a common comorbidity in epilepsy patients. Mutations in *Ank3*, encoding ankyrin G, have been found in bipolar disorder (Ferreira, O'Donovan et al. 2008). Recent work has shown hypoexcitability of parvalbumin positive interneurons in an *Ank3* mutant mouse model of bipolar disorder that may link epilepsy and mood disorders (Lopez, Wang et al. 2016). Because ankyrin G is a VGSC $\beta 1$ interacting protein and reductions in VGSC function in axons are proposed to underlie hypoexcitability of parvalbumin positive interneurons in this model, it is feasible that β subunits play a role in this connection.

Conclusion

The VGSC β subunits are key players in the development of excitability in the nervous system (Fig. 2). β subunits are more than simple accessories of the pore-forming α subunits and instead confer several unique properties to VGSCs that allow precise control of excitability in a cell type specific manner. β subunit multifunctionality allows for crosstalk between VGSC and VGKC function in the CNS and PNS. The cell adhesive properties of β subunits link neuronal development to excitability. Sodium current impacts brain structural development through β subunit dependent pathways and defects in structure resulting from β subunit gene deletion can drive hyperexcitability. These important properties implicate the VGSC β subunits in the mechanisms and potential treatments of several diseases of the nervous system. The cell type dependent properties and unique pharmacology of β subunits are also emerging as informative research tools to study disease mechanisms and to develop novel therapeutics. While the term channelopathy implies defects in the pore-forming subunit of the VGSC signaling complex, the non-pore-forming components are also critical in physiology and disease.

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Highlights

- Sodium channel β subunits are not merely accessories to α subunits.
- β subunits are channel modulators, cell adhesion molecules, and transcriptional regulators.
- Mutations in β subunit genes result in neurological disease.
- β subunit-linked diseases broaden the term ‘channelopathy’ beyond defects in ion conduction.

Figure 1. Topology of the voltage-gated Na⁺ channel α and β subunits

VGSCs contain a pore-forming α subunit consisting of four homologous domains of six transmembrane segments (Catterall 2012). Segment 4 contains the voltage sensor. VGSCs also contain one or more β subunits. $\beta 1$, $\beta 2$, $\beta 3$, and $\beta 4$ contain an extracellular immunoglobulin (Ig) loop, transmembrane domain, and an intracellular C-terminal domain (O'Malley and Isom 2015). $\beta 1B$ also contains an Ig loop, but has a different C-terminus lacking a transmembrane domain, and is thus a soluble, secreted protein (Patino, Brackenbury et al. 2011). $\beta 1$ contains a tyrosine phosphorylation site in its C-terminus (Malhotra, Thyagarajan et al. 2004) ψ , glycosylation sites. $\beta 1$ and $\beta 3$ are non-covalently linked to α , whereas $\beta 2$ and $\beta 4$ are covalently linked through disulfide bonds (O'Malley and Isom 2015). Figure was reproduced from (Brackenbury and Isom 2011).

Figure 2. A multitude of functions of VGSC β subunits in the nervous system

β subunits modulate VGSC function and trafficking to the cell surface (Brackenbury and Isom 2011). Sequential cleavage of β subunits by BACE1 and γ secretase alters transcription, including that of VGSC α subunits, and releases the Ig loop (similar to β 1B) as an extracellular signaling molecule (Wong, Sakurai et al. 2005, Gersbacher, Kim et al. 2010, Patino, Brackenbury et al. 2011). β subunits alter the AIS localization of specific α subunits and can mediate clustering of α subunits (Brackenbury, Calhoun et al. 2010, Namadurai, Balasuriya et al. 2014). Loss of β 1 expression alters axonal fasciculation and pathfinding (Brackenbury and Isom 2011). β 1 subunits at nodes of Ranvier associate with α subunits, likely modifying their function. β 1 in paranodes is engaged in heterophilic cell adhesive complexes independently of VGSC α subunits (Kazarinova-Noyes, Malhotra et al. 2001, McEwen and Isom 2004). Loss of β 2 ameliorates symptoms of multiple sclerosis, likely through axonal-oligodendrocyte interactions (O'Malley, Shreiner et al. 2009). α subunits in the absence of β 2 or β 4 fail to enrich in synaptosomes, suggesting roles in VGSC expression at the synapse (Schmidt, Rossie et al. 1985, Schmidt and Catterall 1986). β 1 facilitates neurite outgrowth at growth cones, requiring I_{Na} (Brackenbury, Calhoun et al. 2010). Mutation of *Scn1b* decreases dendritic arborization, influencing neuronal input

resistance and excitability (Reid, Leaw et al. 2014). Finally, $\beta 1$ is upregulated in reactive astrocytes (Aronica, Troost et al. 2003).