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REVIEW

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Voltage-gated Na^+ channels: multiplicity of expression, plasticity, functional implications and pathophysiological aspects

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Abstract Voltage-gated Na^+ channels (VGSCs) are well known for mediating regenerative cell membrane depolarization and conduction of electrical signalling in nerves and muscles. However, VGSCs may also be expressed in traditionally "non-excitable" cell types, including lymphocytes, glia, fibroblasts and metastatic cancer cells of epithelial origin. Both the diversity and modulation of VGSC expression are far more complex than was initially apparent. There are at least 10 different genes that encode the α-subunits of VGSCs. Since VGSCs can contribute to a range of human disease conditions, it is important to understand both the control and consequences of VGSC functioning and how these aspects may be altered under pathophysiological conditions. Such mechanisms can be at the transcriptional, pre-translational or post-translational levels. This article reviews recent literature that has contributed to our understanding of how individual VGSC subtypes can generate their unique physiological signatures within different cell types. We also highlight emerging areas of interest, in particular, the finding of multiple expression of individual VGSC subtypes within single cells, the generation of alternative splice variants and the increasingly complex set of mechanisms of plasticity through which individual VGSC subtypes may be subtly controlled, including intracellular trafficking of VGSC protein.

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Introduction

Voltage-gated Na^+ channels (VGSCs) are glycosylated membrane-spanning proteins that permit rapid influx of Na^+ in response to membrane depolarization. VGSCs are composed of a "central" α-subunit (~260 kDa) that can be associated with accessory subunits, β_1–β_4 (33–38 kDa), which can modulate functional activity including interaction with cytoskeletal and extracellular matrix proteins (Catterall 2000; Isom 2002; Morgan et al. 2000; Yu et al. 2003). The VGSCα protein contains four repeat domains (D1–4), each of which is composed of six transmembrane segments (S1–6). Whilst the VGSCα subunit is sufficient to form a "functional" channel, VGSCs normally occur as heteromers coupling with one or several auxiliary β subunits (reviewed by Catterall 2000).

Expression of VGSCs is common in classically "excitable" cells within the central nervous system (CNS), peripheral nervous system (PNS) and muscle, where they perform several important functions, especially generation and conduction of action potentials (Hille 1992). However, it has become increasingly clear that VGSCs may also be expressed in traditionally "non-excitable" cell types, including glia (Chiu et al. 1984), lymphocytes (DeCoursey et al. 1985), osteoblasts (Black and Waxman 1996), fibroblasts (Bakhramov et al. 1995), endothelial cells (Gordienko and Tsukahara 1994) and, more recently, metastatic cancer cells of epithelial origin (e.g. Fraser et al. 2002; Grimes et al. 1995; Laniado et al. 1997), where their functions are not so well understood.

It has been stated of VGSCs that "where they do exist..., one is more impressed with their similarity of function than with the differences" (Hille 1992). Certainly, all VGSCs share the same basic biophysical

processes of Na^+ permeation and selectivity, channel activation and inactivation. Many of the amino acid sequences (or "design motifs") that underlie these mechanisms have been identified by biochemical and molecular studies (Fig. 1A). Other VGSCα regions that indirectly modulate these mechanisms include glycosylation, phosphorylation and docking sites for interactions with other proteins (Fig. 1A).

It is now apparent that VGSCs are far more complex than was initially believed and, to date, 10 different genes have been identified that encode $Na_v1.1$–$Na_v1.9$ and Na_x VGSCα subunits. All of these share the same overall molecular architecture but with variable amino acid sequences (reviewed by Plummer and Meisler 1999). When expressed, these VGSCα subtypes have been found to possess subtly different electrophysiological and pharmacological properties. All 10 genes share several highly conserved design motifs (e.g. positively charged S4 voltage sensors, S5/S6 pore-forming regions, D3–D4 linker involved in fast inactivation), but all possess unique amino acid sequences in regions associated with channel modulation (Fig. 1B). These sequence differences probably result in the observed variability in electrophysiological properties, and additionally provide diverse possibilities for protein–protein interactions, that may ultimately endow each VGSC subtype in vivo with a unique "physiological signature".

Furthermore, many of the VGSCαs can be alternatively spliced, thereby adding, deleting or changing amino acid sequences that are responsible for particular electrophysiological activity or protein–protein interaction (Fig. 1C). Individual VGSCα subtypes can thus be expressed in a variety of isoforms that are likely also to be functionally distinct.

Finally, multiple functional VGSCα subtypes and isoforms may occur in single cells, both traditionally "excitable" and "non-excitable". Multiple VGSCα expression may be the consequence of different VGSCαs fulfilling distinct roles. In fact, evidence in "excitable" cells suggests that this multiplicity of expression represents an additional level of functional diversity, allowing even further "fine-tuning" of channel output in response to specific and dynamic requirements of a cell's functional state (Waxman 2000).

In view of the fact that VGSCs may contribute to a multitude of human diseases ("channelopathies") either through genetic mutation (e.g. periodic muscle paralysis, long QT syndrome and epilepsy) or dysregulation of channel gene expression (e.g. chronic pain, multiple sclerosis and cancer) (reviewed by Meisler et al. 2002; Waxman 2001a, 2001b), it is important to gain a detailed understanding of the diversity of VGSC function and modulation. This article is not an exhaustive review of the literature; many excellent recent reviews highlighting different aspects of VGSCs exist (e.g. Cantrell and Catterall 2001; Catterall 2000; Catterall et al. 2003; Dib-Hajj et al. 2002a; Goldin 2001, 2002; Meisler et al. 2002; Plummer and Meisler 1999; Waxman 2001a, 2001b; Waxman et al. 2000; Yu and Catterall 2003). Instead, we have aimed to update the reader on the recent research that has helped define ways in which each VGSC subtype and isoform may differ functionally from each other and how these differences (in some cases very subtle) may contribute to its unique physiological signature. As a consequence, unravelling the complexities surrounding VGSC expression/functioning might provide us with new opportunities to treat particular disorders involving specific VGSCs.

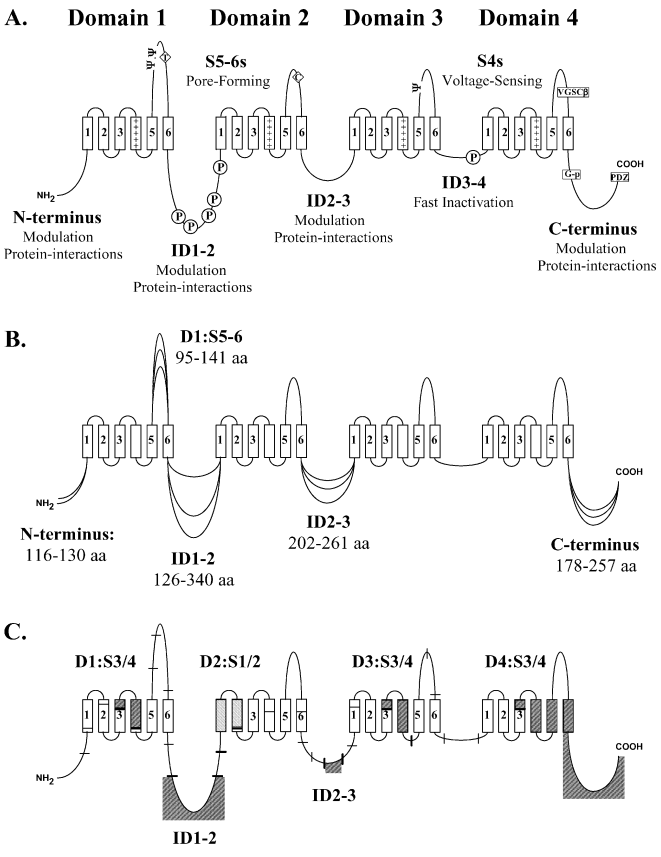

Fig. 1 Schematic diagrams of VGSCα subunit transmembrane folding (**A–C**) showing the four domain structure (D1–4) each domain being composed of the six transmembrane segments (S1–6). **A** Important regions of the protein associated with ion pore formation (S5–6s), voltage-sensing (S4s) and fast channel inactivation (ID3–4), and the VGSCα modulatory regions that modify the activity of these important regions. The major mechanisms for modulation are shown: glycosylation sites (Ψ) phosphorylation sites (P) and known protein binding sites (*boxed*) for VGSCβs, G-proteins (G-p) and PDZ proteins. Locations of toxin-binding sites are also shown for TTX (T) and μ-contotoxin (C). **B** Major areas of difference between the 10 VGSCα subtypes with the range of sizes (in amino acids) of each region in the different VGSCαs. Multiple interdomains (IDs) or extracellular regions represent subtypes of different sizes. **C** Major areas of the VGSCα protein (*dark hashed*) which can be alternatively spliced at the indicated exon boundaries (*thick black bars*) to yield diverse VGSCα isoforms from a single VGSCα gene. Regions where splicing has been documented but appears to be less common are also shown (*light hashed*). Conserved exon boundaries which have not been found to be positions of alternative splicing are also indicated (*thin black lines*)

Functional characteristics of VGSCα subtypes

VGSC subtypes have been characterized by their biophysical characteristics (e.g. activation/inactivation kinetics) and their pharmacological properties (e.g. sensitivity to toxins). Importantly, recent research has indicated that VGSCs possess additional features that can further differentiate individual subtypes.

Biophysical properties

Activation/inactivation kinetics

To understand particular, distinct physiological signatures of individual VGSCs, a detailed characterization of the kinetics is essential. Elucidation of "true" channel kinetics in isolated cells has been difficult, however, because of possible "contamination" by expression of several subtypes of VGSC in their natural environment (e.g. Cummins et al. 1999). Thus, many research groups have expressed individual VGSCα subunits in *Xenopus* oocytes (e.g. Smith and Goldin 1998), mammalian cells (e.g. Cummins et al. 1998) and have made use of natural null-mutants (e.g. Burgess et al. 1995) and transgenic technology (Akopian et al. 1999b). Such work has demonstrated that most VGSCαs ($Na_v1.1$–$Na_v1.4$, $Na_v1.6$, $Na_v1.7$) activate at around −40 mV and have fast inactivation kinetics. However, a subset of VGSCαs ($Na_v1.5$, $Na_v1.8$, $Na_v1.9$) activate at more hyperpolarized potentials (−60 mV or more negative) and possess slower inactivation kinetics. An additional putative VGSC gene product (Na_x) has, to date, not been demonstrated to produce a functional channel (Akopian et al. 1997).

It is important to consider the possible functional consequence(s) of different channel kinetics. For example, expression of $Na_v1.7$, a channel normally associated with the dorsal root ganglion (DRG), in isolation in HEK cells, resulted in Na^+ channels that activated in response to slow depolarizations close to the resting potential (Cummins et al. 1998). This resulted from slow closed-state inactivation and could influence the integrative and firing properties of neurones.

Work is in progress to characterize the molecular regions of the VGSCαs that dictate their individual activation/inactivation characteristics (previously reviewed by Catterall 2000). Recent results have indicated that the first two inter-domain cytoplasmic loops (ID1–2 and ID2–3) alter VGSC activation in a subtype-specific manner (Bennett 2001). For example, these regions are important in determining the activation voltage of $Na_v1.5$ but not $Na_v1.4$. In addition, the first 100-amino-acid stretch of the C-terminal region could generate the differences found in current decay between $Na_v1.4$ and $Na_v1.5$ (Deschenes et al. 2001), whilst a single residue within D2:S5–S6 differentiated between $Na_v1.4$ and $Na_v1.5$ slow inactivation (Vilin et al. 2001). Thus, replacing valine-754 in $Na_v1.4$ with isoleucine from the corresponding position (891) in $Na_v1.5$ altered steady-state slow inactivation characteristics of $Na_v1.4$ to resemble that of $Na_v1.5$. Amino acid residues lining the outer pore have also been implicated in channel gating. For example, for rat $Na_v1.4$, replacement of alanine-1529 by aspartic acid in the putative selectivity filter region of D4 enhanced entry into an ultraslow inactivated state (Hilber et al. 2001).

Pharmacological properties

A variety of toxins have been used as pharmacological tools to distinguish individual VGSCα subtypes. The differing sensitivity of VGSCαs to the guanidines tetrodotoxin (TTX) and saxitoxin (STX) has been particularly well characterized (reviewed by Blumenthal and Seibert 2003; Cestèle and Catterall 2000). Thus, most VGSCαs ($Na_v1.1$–$Na_v1.4$, $Na_v1.6$, $Na_v1.7$) are blocked by nanomolar concentrations of TTX (TTX-S). However, a subset of VGSCαs ($Na_v1.5$, $Na_v1.8$, $Na_v1.9$) shows sensitivity to TTX only at micromolar concentrations (TTX-R). This difference in sensitivity is due to an aromatic residue (denoted **X**) in the pore region of DI:S5–S6 (TMQD**X**WE). Thus, TTX-S VGSCαs possess an aromatic (tyrosine or phenylalanine) residue at this position, whereas TTX-R VGSCαs have a polar residue (cysteine or serine).

More recently, the μ-conotoxins (including GIIIA and PIIIA) have been shown to distinguish $Na_v1.2$ and $Na_v1.4$ from $Na_v1.7$ (Safo et al. 2000). Cummins et al. (2002) have determined that a serine-to-leucine exchange (at amino acid 729) in the D2:S5–S6 region could explain the higher sensitivity of rat $Na_v1.4$ compared to human $Na_v1.4$ to GIIIA and B μ-conotoxins. Differences have also been noticed between individual VGSCα subtypes in the effects of toxins that promote channel activity. Thus, Kondratiev et al. (2003) demonstrated that BmK 11(2), a 7216 Da polypeptide toxin purified from the venom of the scorpion *Buthus martensii Karsch*, increased the peak current amplitude of $Na_v1.4$ and the VGSC in the neuroblastoma cell line N1E-115, but reduced the $Na_v1.5$ current. In addition, type B brevetoxins showed lower affinity for $Na_v1.5$ compared with $Na_v1.4$ (Dechraoui and Ramsdell 2003), whilst the type B brevetoxin PbTx-3 also showed low sensitivity to $Na_v1.7$ (Fraser et al. 2003). There are many other less well-characterized compounds that modulate VGSCs for which studies are ongoing (e.g. batrachotoxin; De Leon and Ragsdale 2003) and it is certainly likely that further neuroactive compounds remain to be discovered.

Regulation and plasticity of VGSCα subtype expression

VGSC expression and activity in the nervous system is highly dynamic, exhibiting significant plasticity of expression, e.g. during development and injury (Wax-

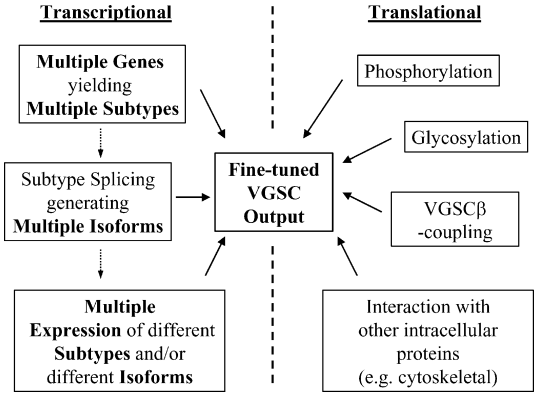

Fig. 2 Schematic diagram summarizing the ways in which the cellular response of the voltage-gated Na^+ channel (VGSC) may be modulated. There are two main levels (transcriptional and translational) at which the ultimate cellular response of the VGSCs may be altered or "fine-tuned". Within either the "transcriptional" or the "translational" level several separate methods for modulating the response may occur and although none can be considered to work in isolation, the events occurring at the transcriptional level may especially be interlinked (*dotted arrows*)

man 2000). Such changes can occur at a number of stages: transcription, pre-translation (e.g. modification of transcript by alternative splicing) and post-translation (e.g. glycosylation, phosphorylation, association with G-proteins, association with cytoskeletal proteins and intracellular trafficking) (summarized in Fig. 2). Such mechanisms may modulate the effective VGSC protein density, determine electrophysiological properties and even the localization of the VGSC within the cell and, ultimately, could result in qualitative and/or quantitative tuning of VGSC activity (e.g. low-frequency versus high-frequency firing states). The "primary" chemical signals inducing such effects include growth factors [e.g. nerve growth factor (NGF), fibroblast growth factor (FGF), glial derived neurotrophic factor (GDNF) and epidermal growth factor (EGF)] and hormones (e.g. androgen and dexamethasone) (see Avila et al. 2003; Cummins et al. 2000; Tabb et al. 1994; Waxman et al. 2000; Zakon 1998; Zur et al. 1995). Some of these effects may be induced through direct interaction with the VGSC (e.g. Liu et al. 2003).

Transcriptional control

The mechanisms controlling VGSCα subunit gene expression are complex but are beginning to be unravelled (reviewed by Marban et al. 1998; Sashihara et al. 1998). At present, limited research has been directed towards identification of the promoter regions controlling VGSC transcription, although information exists for $Na_v1.2$ (Schade and Brown 2000), $Na_v1.5$ (Sheng et al. 1994; Zhang et al. 1999) and Na_x (Gautron et al. 2001). VGSC transcription can be affected by numerous stimuli, including injury (e.g. Black et al. 1999; Iwahashi et al. 1994), electrical activity (e.g. Sashihara et al. 1994), growth factors such as NGF (e.g. Black et al. 1997) and hormones such as androgen (Tabb et al. 1994). Effects may be both cell-type and VGSCα subtype-specific. For example, NGF induced expression of $Na_v1.7$ in PC12 cells (ToledoAral et al. 1995) whilst it upregulated $Na_v1.8$ but downregulated $Na_v1.3$ in DRG neurones (Black et al. 1997).

Transcription factors are fundamental determinants of gene expression. Only one transcription factor (a transcriptional repressor) has so far been shown to affect the specific expression of a single VGSCα subtype (Chong et al. 1995). Thus, expression of $Na_v1.2$ is restricted to rat neurones by REST, which binds to a silencer element (RE-1) in the promotor of the VGSCα gene. REST is expressed in many different rat tissues, where it inhibits $Na_v1.2$ expression (Chong et al. 1995). However, REST is only a part of the repressor mechanism and its function is dependent upon the binding of corepressor proteins, CoREST and mSin3A, to its carboxyl and amino termini, respectively (Andres et al. 1999; Grimes et al. 2000). An RE-1-like silencing element has also been found in the Na_x promoter, indicating that REST may control the expression of at least one other VGSCα subtype. In addition, preliminary promoter analyses suggested that many more transcription factors controlling both general and specific VGSCα gene expression, including Sp1, MyoD and POU-family homeobox proteins such as brn-2 and brn-3, will probably be identified in the near future (J.K.J. Diss, unpublished observations).

Alternative splice variants

The potential for functional diversity of the VGSCα subtypes is increased considerably by alternative mRNA splicing of several of the domains of specific subtypes (Fig. 1C). In this way, several slightly different VGSCαs with substituted modulatory domains can be generated from one VGSCα gene, probably permitting further fine-tuning of VGSC activity. To date, five major sites for alternative exon usage have been identified, enabling inclusion, exclusion or substitution of amino acid residues in modulatory regions. Importantly, studies of alternatively spliced VGSCα isoforms have also revealed how this mechanism is used extensively to generate transcripts which, if translated, would code for VGSCα proteins with major alterations to the conserved transmembrane structure (Fig. 3). Furthermore, levels of these grossly altered VGSCαs are regulated by a variety of stimuli, e.g. cAMP (Oh and Waxman 1998). To date, the role of truncated VGSCα isoforms have not been elucidated but could involve the following: (1) functional association with other altered VGSCαs; (2) a "fail-safe" mechanism producing truncated proteins that could prevent the synthesis or activity of full-length protein; (3) mis-spliced transcripts at sites of alternative splicing which could lead to disruption of the open reading frame, thereby introducing a premature stop position to ensure

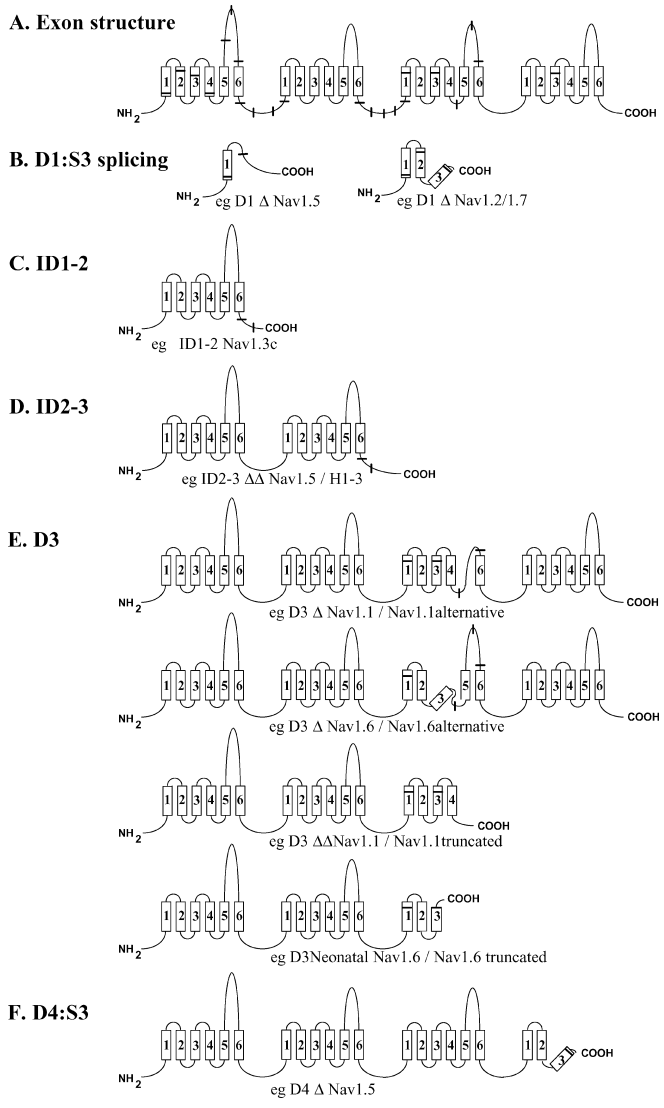

Fig. 3A–F Schematic representations of VGSCα proteins with major alterations to the conserved transmembrane structure predicted to be synthesized from currently known alternatively spliced VGSCα isoforms. **A** Exon structure showing conserved exon boundary positions (*thick black bars*) in all 10 VGSCα subtypes superimposed upon the idealized VGSCα secondary structure. Examples of highly truncated or grossly distorted products arising from: (**B**) domain 1:segment 3 (D1:S3) splicing; (**C**) interdomain 1–2 (ID1–2) splicing; (**D**) interdomain 2–3 (ID2–3) splicing; (**E**) domain 3 (D3) splicing; (**F**) domain 4:segment 3 (D4:S3) splicing at the indicated exon boundaries (*thick black bars*), and named examples are shown. In all cases, one "△" symbol represents one missing exon whilst two consecutive "△" symbols represent two adjacent exons missing

yielding of nonsense, non-functional VGSCs. To date, the following VGSC splice variants have been found:

Domain 1:segment 3 (D1:S3) adult and neonatal isoforms

Developmentally controlled splicing of two alternative exons resulting in two possible transcripts was first described for $Na_v1.2$ and $Na_v1.3$ (Gustafson et al. 1993; Lu and Brown 1998; Sarao et al. 1991). These isoforms differ by only one amino acid in the D1 region of the VGSCα, in close proximity to the voltage-sensing S4. The negatively charged aspartate residue of the adult subtype of both genes is substituted for a neutral residue in the neonatal form: asparagine in $Na_v1.2$, serine in $Na_v1.3$. The effect of this substitution on channel function is not clear, but its conservation across different VGSCα genes and species (rodent and human) would suggest that it is significant. Indeed, the neonatal form of $Na_v1.2$ shows a slightly hyperpolarized activation and steady-state inactivation voltages compared to the adult isoform (Auld et al. 1990). Alternative splicing of D1:S3 neonatal and adult exons has also been found for three other VGSCαs: $Na_v1.5$ (J.K.J. Diss, S.P. Fraser, M.B.A. Djamgoz, unpublished observations), $Na_v1.6$ (Plummer et al. 1998) and $Na_v1.7$ (Belcher et al. 1995), although whether this splicing is developmentally regulated has yet to be determined.

Transcripts derived by the process of exon-skipping (the non-inclusion of any exon coding for a particular part of the protein) have also been identified for Nav1.2, $Na_v1.5$ and $Na_v1.7$ (Fig. 3B) (Diss et al. 2001 and unpublished observations). These isoforms, if translated, would code for highly truncated VGSCαs possessing less than one transmembrane domain. At present, it is unclear whether these transcripts have a functional role or simply represent "junk" mRNAs.

Interdomain 1–2 (ID1–2) isoforms

Splicing of VGSCαs which have the longest interdomain 1–2 (ID1–2) regions (314–340aa; Fig. 1B), including $Na_v1.1$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$, has been reported to result in two $Na_v1.1$ isoforms (1 and 1A), four $Na_v1.3$ isoforms (3, 3A, 3B, 3C), two isoforms of $Na_v1.6$ (8 and 8A) and two isoforms of $Na_v1.7$ (Dietrich et al. 1998; Klugbauer et al. 1995; Plummer et al. 1998; Sangameswaran et al. 1997; Schaller et al. 1992; ToledoAral et al. 1997). ID1–2 splicing can have dramatic functional implications, occurring as it does in a region with potential phosphorylation sites. For example, inclusion of a short exon not present in the "normal" rat $Na_v1.3$ channel would yield the 3B form that introduces two additional potential protein kinase C phosphorylation sites (see Dietrich et al. 1998).

As for D1:S3, there is evidence that some of the splice forms generated in ID1–2, for example $Na_v1.3C$, would also code for highly truncated VGSCα proteins, here possessing just one complete transmembrane domain (Fig. 3C). Unlike D1:S3 splicing, however, truncated isoforms are a consequence of the inclusion rather than skipping of short exons. As yet, it is not known whether the $Na_v1.3C$ isoform has a functional role.

Interdomain 2–3 (ID2–3) isoforms

$Na_v1.5$ and $Na_v1.8$ have extended ID2–3 regions that are at least 40 amino acids longer than most other VGSCαs. An alternatively spliced isoform of $Na_v1.5$ (H1–2) with

an ID2–3 reduced by 53 amino acids, as a consequence of the skipping of a short exon, has been described in mouse heart, rat brain and an embryonic hippocampal precursor cell line, HiB5 (Korsgaard et al. 2001; Zimmer et al. 2002c). In HiB5 cells, this $Na_v1.5$ splice form was found to have more negative voltages of both activation and steady-state inactivation compared with the ''normal'' isoform, and had a 10-fold lower sensitivity to TTX (Korsgaard et al. 2001). However, when this isoform was heterologously expressed in HEK-293 cells, no significant difference compared to the ''normal'' $Na_v1.5$ isoform was apparent (Zimmer et al. 2002c). Additionally, another distinct $Na_v1.5$ isoform (H1–3), generated by the skipping of an adjacent short exon to that yielding H1–2, did not form functional channels when expressed in HEK-293 cells (Zimmer et al. 2002c). If translated, this isoform would code for a protein possessing only two complete transmembrane domains (Fig. 3D).

Domain 3 (D3) isoforms

Alternate splicing of $Na_v1.6$ (at exon 18) in pufferfish, mouse and human resulted in the translation of either a full-length or a highly truncated, two-domain VGSCα protein (Oh and Waxman 1998; Plummer et al. 1997; Schaller et al. 1995). This splicing appeared to be developmentally regulated and tissue specific, the transcript coding for the truncated protein being expressed in foetal brain and non-neuronal tissues. Cells predominantly expressing this isoform did not exhibit any VGSC current and hence it is generally believed that this isoform is functioning as a ''fail-safe'' mechanism to prevent the functional expression of VGSCs (Plummer et al. 1997).

Three other VGSCα isoforms, derived from $Na_v1.1$ and $Na_v1.6$, have been found in rat and human (Diss et al. 2001; Oh and Waxman 1998). These isoforms are generated by exon-skipping events and would code for grossly truncated or architecturally disturbed VGSCαs (Fig. 3E). The functional roles for these isoforms are presently unclear.

Domain 4:segment 3 (D3:S4) isoforms

Jeong et al. (2000) recently identified an alternative splice variant of $Na_v1.9$ located in the conserved intron position in D4:S3. Splicing would result in production of VGSCα protein truncated at D4:S3, but no data are presently available concerning tissue/developmental regulation of expression. Interestingly, Gellens et al. (1992) detected a human $Na_v1.5$ transcript with a 19-nucleotide insert at the D4:S3 splice site, which, if translated, would also code for a VGSCα protein truncated at D4:S3 (Fig. 3F).

Other splicing events

Trans-splicing, whereby (1) exons are present in more than one copy in mRNA transcripts despite existing as single copies in the genome, or (2) exons are spliced together in a different order than that in which they are present in the gene, most likely account for the $Na_v1.8$ splice variant, termed ''SNS-A'', detected by Akopian et al. (1997) in DRG neurones. SNS-A contained an exact repeat of exons 12–14, encoding part of ID1–2, and D2:S1–3. Furthermore, NGF and the functional expression of VGSCs increased the level of the SNS-A transcript in DRG neurones several-fold in vitro and in vivo. SNS-A did not successfully express as a VGSC in *Xenopus* oocytes, and its functional significance is presently unknown.

An alternative transcript of $Na_v1.9$ ($Na_v1.9b$) has also been detected in rat DRG and trigeminal ganglion. $Na_v1.9b$ was predicted to produce a truncated protein due to a frame-shift, introduced by the new sequence of exon 23c (E23c). It has been postulated that E23c might have evolved from the conversion of an intronic sequence (Dib-Hajj et al. 2002a, 2002b). Although a functional role for $Na_v1.9b$ has yet to be established, splicing involving intron-to-exon conversion may be yet another way for VGSCs to acquire novel characteristics.

Clearly, a wide variety of alternative splicing mechanisms are possible for VGSC subtypes and these can result in proteins of considerable size difference. Such isoforms may be physiologically significant, however, as in the case of the single VGSCα gene found in insects, alternative splicing of which can generate pharmacologically distinct sodium channels (Tan et al. 2002). In addition, a VGSC from *Bacillus halodurans*, encoded by only one six-transmembrane domain, was found to be functional when expressed in CHO-K1 cells (Ren et al. 2001). At persent, the mechanism(s) controlling splicing of VGSC genes is not known but could include the membrane potential (Xie and Black 2001), growth factors (Akopian et al. 1999a) and phosphorylation (Shipston 2001).

Post-translational modulation of VGSC activity

Glycosylation

All VGSCαs possess numerous potential extracellular glycosylation sites located mainly in the putative pore-lining regions of D1 and D3 (e.g. Bennett, 2002; Marban et al. 1998). Of these, five are universally conserved, supporting the hypothesis that carbohydrate (mostly sialic acid) plays an essential role in VGSC structure and localization/cell surface expression. However, the extent of this is very much subtype-specific. $Na_v1.1$–$Na_v1.4$ are heavily glycosylated (15–30%), whilst $Na_v1.5$ and $Na_v1.9$ are only about 5% carbohydrate (Marban et al. 1998; Tyrrell et al. 2001). Modulation of glycosylation can also differentially affect electrophysiological properties of individual channels. For example, removal of sialic acid from $Na_v1.4$ using neuraminidase caused a depolarizing shift in both activation and inactivation voltages (Bennett et al. 1997), whilst for $Na_v1.5$ there was a depolarizing shift of activation but a hyperpolar-

izing shift in inactivation (Zhang et al. 2003). Recently, it has been demonstrated that glycosylation may be developmentally regulated (e.g. Castillo et al. 2003; Tyrrell et al. 2001). Thus, deglycosylation of $Na_v1.9$, expressed in neonatal small-diameter neurones of DRG and trigeminal ganglia, reduced the molecular weight to the corresponding level of the channel found in adult cells and also shifted the midpoint of steady-state inactivation to a more depolarized value, reminiscent of the channel in adult DRG (Tyrrell et al. 2001). Altered glycosylation has also been implicated in VGSC dysfunction (e.g. cardiac arrhythmia, neuropathic pain) (Ufret-Vincenty et al. 2001; Zhang et al. 2003).

Phosphorylation

The effects of phosphorylation, and its molecular mechanisms on the functioning of VGSCs, represent an extensive field and have been reviewed before (Bevan and Storey 2002; Cantrell and Catterall 2001; Carr et al. 2003). In brief, VGSCαs can be phosphorylated by the major kinases, including cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC), calcium calmodulin kinase II (CAM kinase II) and tyrosine kinase.

Phosphorylation of VGSCαs by PKA and PKC occurs at two major intracellular sites: D1–2 and D3–4 loops. All VGSCαs possess multiple potential PKA phosphorylation sites, but in vitro studies indicate that only a small proportion of these are actually utilized (Murphy et al. 1996). Thus, PKA only typically modulates the function of the VGSCαs that possess numerous potential PKA sites in their extended D1–2 cytoplasmic linker, i.e. $Na_v1.1$–$Na_v1.3$ and $Na_v1.5$ (Smith and Goldin 1992). Importantly, phosphorylation of VGSCαs by PKA can be subtype-specific. For example, PKA phosphorylation reduced the current amplitude of $Na_v1.2$ without affecting gating, whilst the effect on $Na_v1.5$ was an increased whole-cell conductance (reviewed by Marban et al. 1998). It has also been reported that PKA can modulate the ionic selectivity of $Na_v1.5$, phosphorylation enabling Ca^{2+} to permeate as readily as Na^+, in what has been termed "slip-mode conductance" (Santana et al. 1998). However, subsequent work has suggested that this effect of PKA may, in fact, involve an L-type Ca^{2+} current and not increased Ca^{2+} permeability of the VGSC (e.g. DelPrincipe et al. 2000; Piacentino et al. 2002).

In contrast to PKA, PKC alters the function of all VGSCαs, largely due to phosphorylation of a conserved serine residue in the D3–4 linker that plays a major role in fast channel inactivation (Murray et al. 1997). Not surprisingly, therefore, PKC increased fast channel inactivation, caused reduced maximal conductance and altered channel gating in a VGSCα subtype-specific manner. On the other hand, KN-62, a CAM kinase II inhibitor, reduced peak Na^+ current and hyperpolarized steady-state inactivation of the VGSC in cerebellar granule cells, but had no effect on $Na_v1.2$ when expressed in CHO cells (Carlier et al. 2000).

Importantly, phosphorylation of VGSCαs by individual kinases does not occur in isolation and recent work has attempted to probe the molecular mechanisms of multiple phosphorylation. For example, for $Na_v1.2$, maximal modulation by concurrent activation of PKA and PKC required phosphorylation at four distinct sites in D1–2, with activation of PKC enhancing the PKA modulatory pathway (Cantrell et al. 2002).

Implicit in protein kinase modulation of VGSC function is the opposing modulation by protein phosphatases. Indeed, VGSCs have been shown to be regulated by receptor-like tyrosine phosphatase β (RPTPβ) and calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase 2B. RPTPβ increased Na^+ currents by slowing inactivation and shifting the voltage dependence of inactivation to a more positive value (Ratcliffe et al. 2000). On the other hand, inhibiting calcineurin upregulated cell-surface VGSCs by both stimulating incorporation of the protein into the plasma membrane and inhibiting internalization (Kobayashi et al. 2002).

VGSCs may also be modulated indirectly through phosphorylation of other proteins. Expression of $Na_v1.7$ VGSCs in bovine adrenal chromaffin cells was downregulated by constitutively phosphorylated/activated extracellular signal-regulated kinase (ERK) (Yanagita et al. 2003). In addition, VGSCs may associate with a kinase anchoring protein-15 (AKAP-145), allowing further interaction with PKA and subsequent channel modulation (Cantrell et al. 1999).

Coupling with β-subunits

Of the four VGSCβ subunits (β_1–β_4) identified to date, each contains an extracellular N-terminal domain with an immunoglobulin-like fold, a single membrane-spanning segment, a cytoplasmic C-terminal region and a signal sequence (Isom 2002; Yu et al. 2003). VGSCβs share limited overall homology: β_1 is most similar to β_3, whilst β_2 is most similar to β_4, sharing 43% and 35% identity, respectively.

β subunits can modulate several aspects of VGSC function, including increasing the channel activation and inactivation rates, shifting the voltage dependence of activation/inactivation, switching the channel gating mode from slow to fast and increasing functional channel expression (reviewed by Isom 2002). "Knockout" mice lacking β_2 subunits showed reduced VGSC expression, altered channel inactivation and increased susceptibility to seizures (Chen et al. 2002).

The exact modulation of VGSCs by β subunit coupling is very much dependent on both α and β subtypes involved. $Na_v1.2$ activity has been shown to be modulated by co-expression with β_1, β_2 and/or β_3 (Isom et al. 1992, 1995; Morgan et al. 2000; Stevens et al. 2001), and $Na_v1.1$ with β_1 and/or β_2 (Smith and Goldin 1998). Expression of β_4 with either $Na_v1.2$ or $Na_v1.4$ caused a

negative shift in activation voltage, which overrode the opposite effects induced by co-expression with β_1 and β_3 (Yu et al. 2003). The β subunits can also mediate interaction with extracellular matrix or cytoskeleton, regulate cellular migration and aggregation (reviewed by Isom 2002). β subunits can also associate with ankyrin, tenascin-C and neurofascin, possibly facilitating specific sub-cellular targeting of the VGSCα subunit (Malhotra et al. 2000; 2002; Ratcliffe et al. 2001; Srinivasan et al. 1998). β subunits can also mediate interaction of VGSCαs through other modulatory molecules, e.g. protein tyrosine phosphatase β (Ratcliffe et al. 2000).

The precise structural motifs responsible for β subunit interactions with the VGSCα have not yet been determined. However, several regions have been implicated, including (1) S5/S6 loops of both D1 and D4 (Makita et al. 1996), (2) D4 extracellular loops D4:S3/S4 and D4:S5/S6 (Qu et al. 1999) and (3) a part of the C-terminal cytoplasmic domain (Meadows et al. 2001). In addition, different VGSCαs may interact with particular regions of the β_1 subunit. For example, $Na_v1.5$ is considered to associate via the membrane anchor of the β_1 subunit, in addition to intracellular or extracellular regions, whilst $Na_v1.2$ interacts via an extracellular region only (Zimmer and Benndorf 2002).

The β1 subunit has been reported to exist in alternative splice forms: β1.2 and β1A (Kazen-Gillespie et al. 2000; Oh and Waxman 1994). However, it is unknown what functional effect these alternative forms may have. It is likely that alternative splicing will be an important mechanism for β subunit functioning, as has been found for the VGSC α subunits, and thus more splice variants are likely to be described in the future.

Interaction of VGSCs with other cellular proteins

Individual VGSC subtypes may acquire additional physiological distinction by their ability to interact with other proteins. Over recent years, the identities of an increasing number of such proteins have become known. For example, Liu et al. (2001) showed that the cell adhesion molecule contactin binds directly to $Na_v1.9$ and recruits tenascin to the protein complex in vitro, whilst Malik-Hall et al. (2003) have identified 28 proteins that may interact with intracellular domains of $Na_v1.8$. These proteins include cytoplasmic elements and linker proteins (e.g. β-actin and moesin), enzymes (e.g. inositol polyphosphate 5-phosphatase and TAO2 thousand and one protein kinase), ion channels (e.g. voltage-dependent anion channel VDAC3V) and membrane-associated proteins (e.g. tetraspanin), as well as motor proteins (dynein intermediate and light chain). Such associations may be important in determining the level of expression of the VGSC, the site(s) of targeting and/or the ultimate cellular response.

Garrido et al. (2003) have recently demonstrated for $Na_v1.1$ that the cytoplasmic loop connecting D2–3 is important in localizing the VGSC to axonal initial segment of rat hippocampal neurones, in a process that involved ankyrin G. In addition, Boiko et al. (2001) have shown that $Na_v1.2$ is localized to the unmyelinated zone of retinal ganglion cells, whereas $Na_v1.6$ is specifically targeted to nodes of Ranvier, in this case through a process involving compact myelin. It is also known that Nr-CAM and neurofascin can regulate ankyrin G and VGSC clustering at the node of Ranvier (Lustig et al. 2001).

The interactions with some proteins may be restricted to certain VGSCα subtypes. For example, $Na_v1.1$ and $Na_v1.2$ associate with the synaptic vesicle protein synaptotagmin, through a binding region in the cytoplasmic D1–2 loop. On the other hand, $Na_v1.4$ lacks the domain necessary for such an association (Sampo et al. 2000). The functional consequences of this is unknown at present, but could include the following: (1) intracellular trafficking the VGSCαs to and from the plasma membrane, (2) control of Na^+-dependent exocytosis or (3) modulation of VGSC function via phosphorylation. In addition, it is known that only two VGSCα subtypes, $Na_v1.4$ and $Na_v1.5$, possess PDZ domains, permitting possible direct interaction with other PDZ proteins (e.g. syntrophin and dystrophin). Such interactions may explain the specific localization of VGSCαs at the neuromuscular junction (reviewed by Caldwell 2000). In addition, through the dystrophin-associated protein complex, VGSCαs can link to the actin cytoskeleton and the extracellular matrix. However, brain VGSCαs, which lack the PDZ consensus sequence, also co-purify with syntrophin and dystrophin, suggesting that other sites of interaction must also occur (Gee et al. 1998).

VGSCs are known to associate with calmodulin in both a Ca^{2+}-sensitive and Ca^{2+}-insensitive manner (e.g. Herzog et al. 2003; Mori et al. 2000). Recent results have indicated that the IQ-motif of the VGSCα interacts with the C-terminal lobe of calmodulin (Mori et al. 2003) and that disrupting this interaction reduced the current amplitude of both $Na_v1.4$ and $Na_v1.6$. In contrast, calmodulin interaction modulated the inactivation kinetics of $Na_v1.6$ only (Herzog et al. 2003).

VGSC–protein interactions can lead to a variety of functional effects. For example, cytoskeletal interaction induces modulation of VGSC peak open probability, persistent activity and activation (Maltsev and Undrovinas 1997; Undrovinas et al. 1995). Contactin increased the functional expression of VGSCs (Kazarinova-Noyes et al. 2001), whilst $Na_v1.5$ interaction with the ubiquitin protein ligase Nedd4 reduced the peak current, possibly through ubiquitination and subsequent endocytosis of the channel protein (Abriel et al. 2000). Ankyrin G plays an essential role in coordinating the physiological assembly of $Na_v1.6$, βIV spectrin and the Ll cell adhesion molecules (L1-CAMs), neurofascin and Nr-CAM at initial segments of cerebellar Purkinje neurones (Jenkins and Bennett 2001), whilst ankyrin G and βIV spectrin help stabilize VGSC clustering at the nodes of Ranvier (Komada and Soriano 2002). An ankyrin-binding motif has recently been determined for

the intracellular loop between D1–2 (Bouzidi et al. 2002; Lemaillet et al. 2003).

Lastly, G-proteins have also been shown to modulate VGSC activity. Thus, $\beta\gamma$ subunits induced persistent VGSC currents by directly binding to the QXXER motif in the cytoplasmic tail of $Na_v1.2$ and slowing channel inactivation (Ma et al. 1997). $Na_v1.1$, $Na_v1.6$ and $Na_v1.7$ also possess this binding motif, indicating that they too may be modulated by these G-protein subunits. In addition, G-protein coupled receptor signalling cascades, working through phosphorylation of the VGSCα subunit, can lead to alterations in channel kinetics with a striking resemblance to that of slow inactivation (Carr et al. 2003). Such a mechanism may be quite widespread, but with possible exceptions. For example, in hippocampal pyramidal neurones, phorbol esters actually slowed the development of slow inactivation (Magee et al. 1998). G-protein coupled receptors have also been implicated in upregulation of $Na_v1.9$ in small-diameter sensory neurones and thus may be important to nociception (Baker et al. 2003).

Intracellular trafficking

A relatively unexplored area to date involves VGSC protein trafficking within the cell. Recent research has investigated the intracellular processing and subcellular localization of $Na_v1.5$ within cardiac myocytes and HEK293 cells (Zimmer et al. 2002a, 2002b). When expressed alone, $Na_v1.5$ was highly expressed within the endoplasmic reticulum (ER) of HEK293 cells. Similar results were found for $Na_v1.5$ distribution in cardiac myocytes (Zimmer et al. 2002a). In co-transfection studies, β_1 but not β_2 colocalized with $Na_v1.5$ and the resulting complex was restricted to the ER (Zimmer et al. 2002b). The mechanisms involved remain unknown, but activation of PKA is known to modulate the trafficking of both $Na_v1.5$ and $Na_v1.8$ from the ER to the plasma membrane in *Xenopus* oocytes (Zhou et al. 2000; Vijayaragavan et al. 2004).

Multiplicity of cellular VGSC expression

Expression of mRNAs for multiple VGSCα subtypes is now known to occur under both physiological and pathophysiological conditions, in both traditionally "excitable" and "non-excitable" cells. For example, several VGSCα subtypes are expressed in the magnocellular neurosecretory cells of the supraoptic nucleus (Tanaka et al. 1999), DRG neurones (e.g. Black et al. 1996), retinal ganglion cells (Boiko et al. 2003; Fjell et al. 1997), glia (Sontheimer et al. 1996), gliomas (Schrey et al. 2002) and breast and prostate carcinoma (Diss et al. 2001; Fraser et al. 2002). In mouse sinoarterial (SA) node, both $Na_v1.1$ and $Na_v1.3$ were present, but $Na_v1.5$ was not. It was hypothesized that $Na_v1.1$ and $Na_v1.3$ are required because their more positive voltage dependence of inactivation would allow them to function at the depolarized membrane potential of SA nodal cells (Maier et al. 2003).

Importantly, different VGSCαs subtypes can be functional within the same cell in both neuronal, e.g. DRG (Cummins et al. 1999), and "non-neuronal" tissues (e.g. Fraser et al. 2002). Thus, both $Na_v1.8$ and $Na_v1.9$ were expressed in small C-type DRG neurones, where together they could modify excitability (Cummins et al. 1999; Vijayaragavan et al. 2001). In the metastatic human breast carcinoma cell line MDA-MB-231, both $Na_v1.5$ and $Na_v1.7$ were expressed (Fraser et al. 2002), and functional VGSC activity enhanced cellular invasiveness (Fraser et al. 2002; Roger et al. 2003).

At present, the functional significance of multiple VGSCα subtype expression within individual cells and tissues is unclear. It has been suggested that, within neurones, multiple VGSCα expression may contribute to functional plasticity by "fine tuning" the response patterns of particular neurones (Waxman 2000). Further to this, the expression of low-level mRNAs for more than one VGSCα subtype in a cell, even in cases where there is little evidence for functional expression, may also allow faster upregulation of particular subtypes, in accordance with changing functional requirements that could result from the cells' dynamic micro-environment.

VGSCα subtypes and pathophysiology

Maladaptive changes in the plasticity of VGSC gene expression can occur in some pathological states both in neuronal (reviewed by Lai et al. 2003) and non-neuronal tissue (e.g. Diss et al. 2001; Fraser et al. 2002; Roger et al. 2003). For example, spinal cord astrocytes switch from TTX-S to TTX-R VGSC expression following injury-induced gliosis (MacFarlane and Sontheimer 1998), whilst expression of normally silent VGSC genes may contribute to multiple sclerosis (reviewed by Waxman 2001b). Transection of spinal sensory neurones can change VGSC expression, including down-regulation of $Na_V1.8$/$Na_V1.9$ and up-regulation of $Na_V1.3$ (reviewed by Waxman 2001b). Even redistribution of $Na_V1.8$ in uninjured axons may contribute to neuropathic pain (Gold et al. 2003).

Expression of multiple VGSCα subtypes may also be relevant in pathophysiological conditions. For example, the rat epithelial prostate cancer cell line MAT-LyLu possesses mRNAs for at least seven different VGSCα subtypes. Of these, the highest mRNA levels found were for $Na_v1.7$, $Na_v1.1$ and Na_x. In comparison, the weakly metastatic counterpart cell line AT-2 showed only low levels of VGSCα mRNA (Diss et al. 2001). However, electrophysiological recordings indicated that the MAT-LyLu cells expressed a single population of inward VGSCs with biophysical/pharmacological characteristics showing similarity to $Na_v1.7$ (Grimes and Djamgoz 1998; S.P. Fraser, unpublished observations). Importantly, the VGSC in the MAT-LyLu cells plays an

important role in several fundamental cellular behaviours linked to cancer metastasis, e.g. invasion (Grimes et al. 1995), motility (Fraser et al. 2003), morphological enhancement (Fraser et al. 1999), galvanotaxis (Djamgoz et al. 2001) and secretory membrane activity (Mycielska et al. 2003).

As well as changes in the VGSCα subtype expressed, pathophysiological conditions may involve re-splicing of a given VGSCα. Thus, kainate-induced seizures in adult rat hippocampus resulted in increased mRNA levels of the neonatal splice forms of $Na_v1.2$ and $Na_v1.3$ (Gastaldi et al. 1997). Interestingly, in the case of metastatic breast and prostate cancer, the VGSCαs expressed have also been found to be in their neonatal splice form (Diss et al. 2001; Fraser et al. 2002). This is in line with "oncofoetal" gene expression (Monk and Holding 2001) and epigenetic control of cancer (e.g. Momparler 2003).

Finally, β subunits may also be important in human disease. For example, a mutation in the β_1 subunit gene has been linked to generalized epilepsy with febrile seizures plus type 1 (Meadows et al. 2002).

Conclusion and future perspective

In conclusion, although VGSCα subtypes show considerable sequence homology and, in fundamental terms, the workings of VGSCs are basically similar in allowing Na^+ influx, each subtype may show functional variations that may be subtle or quite marked. Such variations may occur initially because of differences in sequence, including those resulting from alternative splicing. Functional diversity has been demonstrated in terms of activation/inactivation kinetics, post-translational modification (i.e. by phosphorylation or glycosylation) or their interaction with particular cellular proteins. The diversity of VGSC expression and the selective expression of different VGSC subtypes and isoforms in specific tissues would strongly suggest that the resulting functional differences may be of major physiological significance. The mechanisms through which such modifications occur are summarized in Fig. 2.

In contrast, our understanding of the precise molecular mechanisms that regulate particular subtype expression and either the "upstream" or "downstream" signalling cascades driven by VGSC subtype-specific expression/activity is very much in its infancy. In addition, the rationale for expression of VGSCs in traditionally "non-excitable" cells, the occurrence and plasticity of multiple VGSCs within single cells and their possible contribution to pathophysiological conditions deserve much more research. In turn, this promises to lay the grounds for new therapeutic opportunities, as drugs designed towards targeting expression and/or activity of individual VGSC subtypes become available.

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