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Review

Mechanisms of voltage-gated ion channel regulation: from gene expression to localization

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Abstract. The ion channel milieu present in a neuron in large part determines the inherent excitability of a given cell and is responsible for the translation of sensory transduction and synaptic input to axonal output. Intrinsic excitability is a dynamic process subject to multiple levels of regulation from channel gene expression to post-translational modifications that influence channel activity. The goal of this review is to provide an overview of some of the mechanisms by which channels can be modified in order to influence neuronal output. We focus on four levels of regulation: channel gene transcription, alternative splicing of channel transcripts, post-translational modifications that alter channel kinetics (phosphorylation), and subcellular localization and trafficking of channel proteins.

Keywords. Ion channels, phosphorylation, channel kinetics, alternative splicing, trafficking, and neural plasticity.

Introduction

The intrinsic properties of a neuron determine the inherent excitability of a given cell and are responsible for the translation of sensory transduction and synaptic input to axonal output. It is this input-output relationship that is the heart of all nervous system activity. The excitability of any given neuron is most attributable to the proteins that are directly responsible for changes in membrane potential: the voltage-gated ion channels. These channels span the plasma membrane and generate ion-selective pores for the movement of charged particles across the membrane that ultimately results in membrane potential fluctuation in excitable cells. Thus it is the combined milieu of voltage-gated channels present, as well as the specific kinetics of the activity of these channels, that ultimately generates the response of a cell to a particular pattern of stimuli.

Intrinsic excitability is not a static state, but rather a dynamic process subject to multiple levels of regulation from channel gene expression to post-translational modifications that profoundly influence ion channel activity. This diversity of mechanisms that underlie plasticity in neuronal output is one of the core features of the malleability of the nervous system. For if the nervous system and its constituent neurons were simply static input-output devices, then dynamic processes such as learning and memory, sensory adaptation, motor coordination, and autonomic regulation of homeostatic processes would not be possible. Thus the flexibility of the individual units of the nervous system emerges to form the complex and adaptable neural networks that give rise to higher functions in all animals. Therefore, plasticity in ion

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channel function is at the very foundation of nervous system function itself.

The role of ion channels in the variability and plasticity of cellular phenotypes and output is due not only to flexibility in channel expression and protein function, but also as a result of the sheer diversity of ion channel subtypes present in the nervous system, or even within a single cell. Ion channels perform their roles by allowing a select group of essentially four ions across the membrane: Na^+, K^+, Ca^{2+}, and Cl^-. Yet the passage of K^+ alone across the membrane is the result of at least 100 different K^+ channel subunits [1]. There are at least 9 different genes that code for the pore forming α-subunits of voltage-gated Na^+ channels in humans [2], and those for the gene family encoding the primary voltage-gated calcium channels also number at least 10 [3, 4]. To this diversity add the fact that a functional channel is ultimately a combination of these α-subunits with a subset of accessory subunits that also display tremendous diversity, and it is not difficult to appreciate the complexity underlying the intrinsic excitability of neurons at all levels of the nervous system.

The goal of this review is to provide an overview of some of the mechanisms by which voltage-gated ion channels can be modified in order to generate plasticity in neuronal output. We focus on four levels of regulation: channel gene transcription, alternative splicing of mRNA transcripts, post-translational modifications that alter channel kinetics (phosphorylation), and subcellular localization and trafficking of channel proteins. As discussed above, the extraordinary diversity of channel subtypes makes a comprehensive review of these topics for each channel type impossible. Rather, we have chosen to focus on general mechanisms of channel regulation rather than the details of how each individual channel is modified. Toward this end, we use the example of voltage-gated sodium channels (VGSCs) throughout each section as a device to illustrate how each level of channel regulation can influence the same channel type in order to create a diversity of neuronal output. In addition, we provide examples from other channel families to emphasize the fact that these are common mechanisms employed in generating plasticity of channel function and neuronal output.

Voltage-gated channel structure as subtrates for plasticity

Voltage-gated channels consist of membrane-spanning proteins that permit the rapid influx and/or efflux of charged ions in response to changes in membrane potential. These channels are composed of a core of a single α-subunit or a multimeric association of α-subunits that largely make up the membrane-spanning pore of the channel complex. While these α-subunits are the focus of this review, virtually all channels are heteromers of the pore-forming α-subunits with accessory subunits involved in anchoring the protein to the plasma membrane or influencing channel kinetics, interactions with cytoplasmic, or cytoskeletal elements. Together these protein subunits determine the kinetics of the ion channel function, i.e. at what membrane potential and how rapidly the channel opens ('activation'), closes ('deactivation and inactivation'), and how much current each channel carries when it is open.

The α-subunits of VGSCs are structurally representative of how most ion channels are organized (see Fig. 1) [5]. The channel proteins themselves are taxonomically described by a numerical system of the primary channel type (e.g. Na_v1 for VGSCs) and a numerical suffix that describes a distinct channel protein. Hence the VGSC proteins are named $Na_v1.1$–$Na_v1.9$. These VGSCs all consist of four repeat domains (D1–4), each of which is composed of six transmembrane segments (S1–S6). The transmembrane segments and the domains they make up tend to be highly conserved and are responsible for the voltage-sensing properties of the channel as well as the pore selectivity. Conversely, the portions of the protein that join together the four repeat domains are areas of diversity between channel types, and contain the majority of the residues of the protein that are involved in post-translational modification and protein-protein interactions. Therefore, it is these 'linker' sequences that are primarily responsible for the diversity of electrophysiological phenotypes that can be observed between different VGSC subtypes.

Transcriptional control of ion channel expression

With regard to gene expression and the functional output of any cell, the governing factors are obviously which gene is expressed, how much of this mRNA is present in the cell (the relative abundance), and how this expression is regulated. The voltage-gated ion channels are no exception, and there are myriad examples of how expression of ion channel genes differs between cell types, or under different environmental conditions, including injury. Beyond cell-type-specific differences in the abundances of which VGSC subunits are expressed, within a given cell type VGSC expression is a dynamic process as well. Indeed, with upwards of 10 different VGSC genes, it would be surprising if variability were not the norm in this situation. Expression of VGSC is known to be

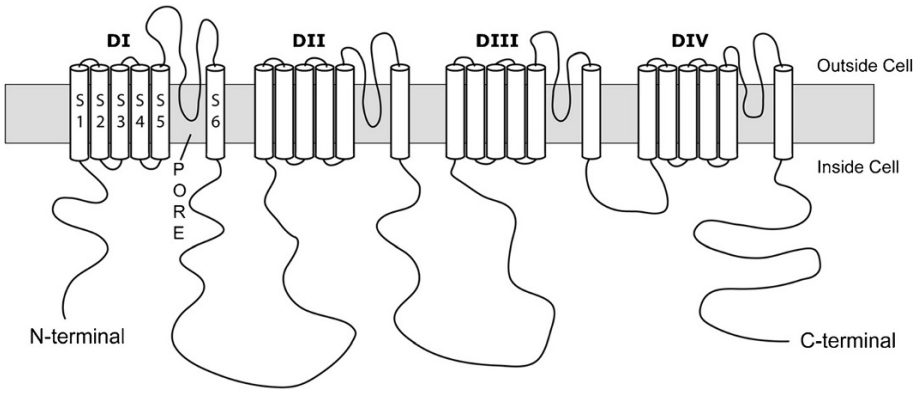

Figure 1. Basic structure of a voltage-gated sodium channel. The α-subunits of VGSCs consist of a single protein with four transmembrane domains (DI–DIV) that surround a Na^+ ion selective pore. Each transmembrane domain consists of six segments (S1–S6), joined by linker segments that vary between VGSC subtypes.

influenced by such diverse factors as electrical activity within the cell [6], injury to the cell [7], trophic factors [8], and hormonal influences [9].

In order to understand how expression of particular voltage-gated channel subunits influences the output of an individual neuron, multiple levels of understanding must be achieved. First, at the level of the single neuron the diversity of channel types that are expressed must be elucidated. For example, in the leech it has been demonstrated that individual neurons not only express more than one VGSC isoform, but do so in a cell-specific pattern of VGSC isoform expression [10]. Few studies have been undertaken at the resolution of single-neuron types [11], but such studies are of great importance. Second, an understanding must be gained of how the mix of a given set of channel subunits gives rise to the overall membrane current. While many channels have been characterized as single subunits in artificial expression systems such as *Xenopus* oocytes [12], it is not known how combinations of multiple channel subunits lead to overall membrane currents. Finally, to come full circle in our understanding, the regulation of channel subunit gene expression must be determined (discussed below).

Changes in channel expression as a result of disease-induced changes in activity

To begin to understand how voltage-gated channel subunit expression is regulated, multiple models have been employed that look at how disease states lead to changes in channel gene expression. For example, a number of studies have asked whether epilepsy models such as pharmacologically induced status epilepticus (SE) result in altered expression of VGSC subunits. Kainic acid-induced SE results in a transient upregulation of mRNAs encoding the $Na_v1.2$ and $Na_v1.3$ α-subunits in the adult rat hippocampus as revealed with *in situ* hybridization [13]. These results were extended with real-time PCR experiments that demonstrated that mRNA levels for $Na_v1.1$ and $Na_v1.3$ are significantly elevated in the hippocampus of double-mutant spontaneously epileptic rats [14]. Furthermore, these changes in expression are not limited to the α-subunits, nor are they necessarily in a single direction. Spontaneously epileptic rats display elevated hippocampal levels of β1-subunit expression [14], while SE induces a transient increase in β2-subunits for 1 h followed by a significant and relatively long-lasting (72 h) decrease in these subunits in the rat hippocampus [15]. Together these results suggest that dysregulation of sodium channel subunit expression leads to alterations in sodium channel activity, and ultimately alters the intrinsic properties of the neurons involved as well as contributing to the underlying pathology of epileptic seizures.

Extensive evidence regarding changes in channel gene expression, particularly in sensory neurons of the dorsal root ganglion (DRG), also has been uncovered in multiple models of neuropathic pain. Five VGSC α-subunits ($Na_v1.1$, $Na_v1.6$, $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$) are expressed at appreciable levels in DRG of adult rats, and the expression of these channels is strongly influenced by nerve injury. $Na_v1.3$ is not typically expressed in DRG cells, but has been found to show a great deal of plasticity, largely upregulation, as a result of sciatic nerve transection [16], chronic nerve constriction [17], and spinal nerve ligation [18]. Conversely, $Na_v1.1$, $Na_v1.6$, and $Na_v1.7$ are downregulated following spinal nerve axotomy [18, 19]. In addition to pain models that employ direct nerve injury, VGSC expression also changes as a result of inflammatory pain. Na_v 1.3, $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$ all have been shown to be upregulated in models of chronic inflammatory pain [20–22], with these changes persisting for at least some weeks [21]. By and large, these changes in VGSC are thought to underlie the increased excitability of DRG neurons following nerve injury, and it is this hyperexcitability that leads to chronic pain symptoms [23].

One study that begins to make the functional connection between VGSC expression and functional

changes in sodium currents combines patch clamp recordings with real-time PCR measurements in a model of SE [24]. This study reveals that pilocarpine-induced SE is associated with a shift in the activation voltage towards hyperpolarized potentials and a shift in the inactivation voltage towards more depolarized potentials in dentate gyrus cells of the rat hippocampus, resulting in increased excitability of these cells. Concurrently, this shift was correlated to a downregulation of β2-subunit expression. Because coexpression of β-subunits is known to shift inactivation voltage in a hyperpolarizing direction [12], these results represent preliminary evidence that down-regulation of β-subunit expression may lead to a depolarizing shift in the inactivation voltage following SE. Similar associative lines of reasoning present strong implications for how changing gene expression of VGSC can lead to hyperexcitability and ultimately chronic pain in DRG axotomy models [7, 25]. However, much finer resolution experiments that combine electrophysiology and gene expression at the single-cell level [26, 27] are needed to strongly implicate changes in channel subunit expression with functional changes that affect the output of neurons following SE. Nevertheless, such models can serve as a valuable starting point for understanding both the effects of altering channel expression, as well as ultimately lead to knowledge regarding the transcriptional regulation underlying these changes.

Mechanisms of transcriptional regulation of ion-channel genes

While the phenomenon of plasticity in expression of voltage-gated channels is well established, knowledge of mechanisms underlying the regulation of this expression is scarce. Thus far, only one transcription factor, a functional repressor element 1-silencing transcription factor (REST or NRSF) has been shown to affect VGSC expression [28]. REST/NRSF was first identified as a repressor of the expression of the neuronal $Na_v1.2$ gene [28, 29]. However, REST/NRSF binding sequences are also found in many other neuronal and non-neuronal genes, and therefore does not act as a specific agent in the expression of voltage-gated channels [30, 31]. Thus the effects of REST/NRSF as a transcriptional repressor are somewhat widespread and not specific to the regulation of voltage-gated ion channels, or even neuronal genes in general.

More recently, exciting evidence has implicated a new mechanism for how voltage-gated channel function can influence gene transcription, and perhaps act in a negative feedback mechanism to influence channel expression itself. In neurons, L-type voltage-gated calcium channels ($Ca_v1.2$) produce a C-terminal fragment that translocates to the nucleus and regulates gene transcription [32]. This C-terminal fragment, dubbed the calcium channel-associated transcription regulator (CCAT), interacts with the transcriptional regulator p54(nrb)/NonO in the nucleus and can activate transcription of endogenous genes that are important for neuronal excitability and synaptic signaling. In particular, this CCAT complex binds to the enhancer of the *connexin 31.1* gene (Cx31.1), responsible for gap junctions and thus electrical synapses in these cells, and directly regulates the expression of Cx31.1. Furthermore, the nuclear localization of CCAT is regulated both developmentally and by changes in intracellular calcium, potentially providing a direct link between neuronal activity and gene transcription. Further evidence suggests that C-terminal cleavage is a general feature of voltage-gated calcium channels [33, 34] and that other members of this family may also be transcriptional regulators [35]. These findings provide evidence that voltage-gated calcium channels can directly activate transcription and suggest a mechanism linking voltage-gated channels to the function and differentiation of excitable cells.

While an understanding of plasticity in ion channel expression and its underlying mechanisms of regulation are slowly emerging, recent work indicates that such regulation of channel expression (and ultimately cellular excitability) can be highly variable from cell to cell, and does not occur independently for single ion channels. Expression levels for a given ion channel can vary 2–4-fold in a particular class of neuron [27], and this level of variability correlates with measurements of membrane conductance [27, 36]. Yet some fixed relationships may exist among ion channels that may play a role in stabilizing neuronal output. Quantitative single-cell PCR measurements of the levels of mRNA for six different ion channels from the same single identified motor neurons demonstrate that multiple ion channels in a single cell type show correlated levels of expression [37]. Further, in six different individually identifiable cell types examined, no two cell types showed the same set of correlated channel expression levels [37]. These results suggest that cell-specific mechanisms exist to co-regulate the expression of voltage-gated channel genes, perhaps to stabilize neuronal output or compensate for altered excitability as has been demonstrated for VGSC as well [38, 39]. Such compensation has been elegantly demonstrated in neurons of the crustacean stomatogastric ganglion, where overexpression of the channel responsible for a transient K^+ current (I_A) leads to a drastic increase in the measurable membrane current, but very little effect on the output of the neuron [26, 40]. This is due to a concomitant and compensatory increase in the

hyperpolarization-activated inward current (I_H), the depolarizing activity of which is thought to act in opposition to that of the hyperpolarizing I_A current. A similar compensation is thought to occur for calcium currents in basal forebrain neurons [41]. Thus in the future it will be important to determine not only the mechanisms by which activity influences transcription of the voltage-gated channel genes, but how multiple channel genes may be co-regulated in order to generate functional neuronal output as well as compensate for changes in activity levels of these cells.

Alternative splicing of ion channel genes

Beyond the simple 'on or off' expression of a particular ion channel gene, alternative splicing enables the same gene to generate multiple mature mRNA types for translation, resulting in multiple channel proteins. The result of this variety is functional diversity, which, in turn, may have consequences on cellular function. This mechanism is evolutionarily conserved across animal phyla and is particularly common in neural genes, suggesting that this is a widespread mechanism for influencing cellular processes [42].

Alternative splicing consists of changes to sequential exons by including or removing them from the mRNA. This process is carried out by spliceosomes, which first recognize intron and exon boundaries [43]. There are different patterns of alternative splicing, which may occur independently or as a combination of events. The most common type of alternative splicing is 'exon skipping' [42], which either includes or excludes a specific exon sequence. In 'mutually exclusive exon splicing', there is more than one alternative exon sequence but only one of the sequences is included in the mature mRNA. In addition to addition or removal of specific exonic sequences, transcription from different promoters for the same gene may also result in splice variants with different 5′ regions [44]. Alternative 3′ sites are also possible due to multiple poly-adenylation regions within a gene [45]. Thus alternative splicing is a complex process with multiple mechanisms to generate diversity of protein function from a single gene (reviewed in detail in [42, 43, 45]).

Recent studies indicate that alternative splicing and tissue cell-type splice variant expression of voltage-gated channels may lead to channels with different properties, including channel kinetics, which therefore result in changes in neuronal output. Further, there is evidence that alternative splicing may itself be regulated by environmental stimuli or cellular activity [43]. Using splice variants of sodium channel genes as examples, the consequences and the functional implications of these changes as a result of alternative splicing will be discussed in this section.

Alternative splicing produces channels with distinct kinetics

Alternative splicing has been shown to result in VGSCs with differing channel kinetics (see Fig. 2). One possible change that may result from alternative splicing includes altered activation states. For example, two splice variants of $Na_v1.5$, hNbR1 and hNbR1–2, differ in the presence or absence of exon 18 as a result of exon skipping. hNbR1, which includes exon 18, has a lower I_{Na} activation, along with lower peak current and average range of I_{Na} than hNbR1–2, which does not include exon 18 [46]. The functional significance of the presence of these splice variants in cells has yet to be investigated, but these results suggest that hNbR1 may be more easily activated and deactivated, leading to increased excitability. The steady-state activation and inactivation of hNbR1–2 are negatively shifted with respect to those of hNbR1, which could cause cells that express this variant to require more stimulation due to a reduction in excitability [46]. In addition, $Na_v1.3$ splice variants that contain variable lengths of exon 12 also show a similar type of channel kinetic difference [47]. Splice variant 12v1 has the shortest length of exon 12 followed by 12v2, 12v3, and lastly 12v4, which contains the full length of this exon. Only the two shortest splice variants show measurable changes in channel kinetic properties. Splice variant 12v2 requires more depolarization in order to be activated, which may result in reduced excitability for cells expressing this variant. Splice variant 12v1 also may lead to less excitable cells, but instead of requiring greater depolarization, these channels inactivate more rapidly. The other two splice variants, 12v3 and 12v4, displayed another possible change in channel kinetic properties as a consequence of alternative splicing, changes in phosphorylation. In addition to the inclusion of longer versions of exon 12, 12v3 and 12v4 contain additional protein kinase C and casein kinase II sites [47]. But the functional significance of these added sites is poorly understood. It is possible that these additional sites may affect channel kinetics via phosphorylation state, as well as influence interactions with other proteins.

In addition, alternative splicing results in changes to channel kinetics as a result of changes in the channel's gating mode. Two splice variants of *scn1ba*, a sodium channel β1-subunit expressed in zebrafish, are designated *scn1ba_tv1* and *scn1ba_tv2*. These splice variants differ in their C-terminal domain, with *scn1ba_tv2* lacking 12 amino acids and a C-terminal tyrosine, which is essential for subcellular localization and channel modulation [48]. Although both splice

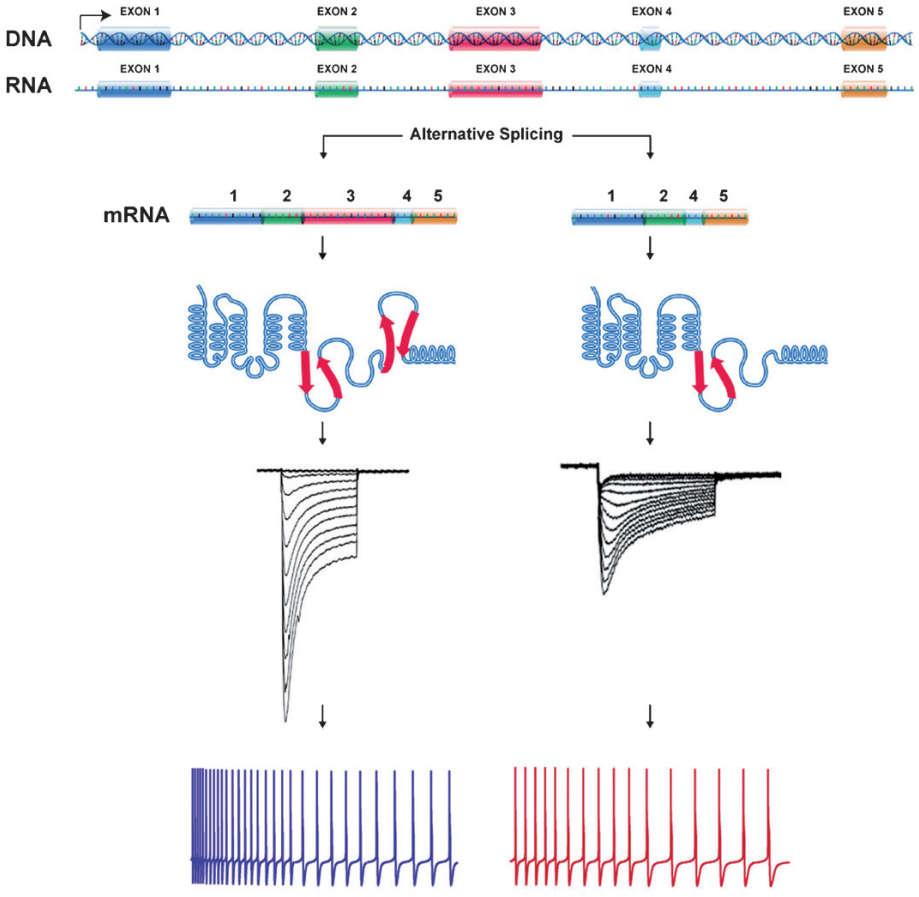

Figure 2. Alternative splicing influences channel kinetics and neuronal output. Alternative splicing results in multiple protein products from a single gene. Although data regarding the effects of splice variants on both ionic currents and firing patterns of single neurons are scarce, in this hypothetical example of exon skipping, the channel on the left has five exons, and results in a larger inward current (black trace) as well as a more excitable neuron (blue trace, bottom). Conversely, the hypothetical channel resulting from the splicing event on the right has a smaller inward current (black trace) and therefore results in a less excitable neuron (red trace, bottom). Adapted from a public domain image courtesy of the National Human Genome Research Center.

variants show similar physiological properties to mammalian β1-subunits, they differ from these β1-subunits by shifting their channel-gating mode from slow to fast, and neither led to a complete channel inactivation or recovery from inactivation [48]. These changes may result in altered cellular activity, but again such changes are not well understood in a biological context. Further examination of these and other kinetic properties due to the presence of alternate splice forms will undoubtedly become an important area of research towards determining how overall cellular activity is modulated.

Tissue-specific distribution of channel variants

Alternative splicing also is regulated in such a way as to result in tissue-specific patterns of expression. Although splice variants may show overlapping expression in different tissues, one splice variant may be expressed exclusively in one tissue while other splice variants are solely expressed in another tissue type. For example, the $Na_v1.6$ gene is expressed in both neuronal and non-neuronal cells. Yet splice variants exist that vary in exon 18, and these resulting splice variants are expressed in different cell types. For three $Na_v1.6$ splice variants (18N, Δ18, and 18A), only two (18N and Δ18) are expressed in utricular hair cells, but 18A is the sole form of $Na_v1.6$ expressed in adult dorsal root ganglion [11].

In other cases, different cell types may express overlapping populations of splice variants, yet differ in the relative abundance of each variant. For the $Na_v1.5$ gene, alternative splicing occurs in the extracellular S3–4 linker on domain 1 (D1:S3) of the protein [49]. Splice form $nNa_v1.5$ is more abundantly expressed in the heart and brain, while of low abundance in the skeletal muscle during neonatal development. Levels of expression in these areas slowly decrease as the animal progresses into adulthood [49]. Splice variants of SCN5A, a cardiac sodium channel gene, also shows this variability in tissue-specific abundance. Splicing occurs in more than one area, at exon 1 or at the 3′ region of SCN5A, to form these variants, which result in different levels of tissue expression. Most notably, splice variant exon 1C is found in relatively high abundance, while the short 3′-UTR mRNA form was expressed in lower levels in the adult heart tissue [49]. The biological significance of different tissue-specific expression of splice variants is not well understood, especially when these splice variants do not differ in other properties, such as channel kinetics [50]. Devel-

opmental regulation of alternative splicing appears to be involved in these situations where splice forms occur with different distribution profiles in different tissues [51–53]. However, the mechanism underlying this differential regulation is not well understood.

Activity-dependent regulation of alternative splicing

Previous examples in this review have shown that alternative splicing may influence cellular activity. This relationship also may be reciprocal, as there is evidence that cellular activity can feed back to regulate alternative splicing. Although the signal transduction pathways are not well understood, such a pathway would lead to the production of splice variants that may improve or help adjust cellular responses to specific environmental changes or stimuli. For example, it was found that kainic acid-induced SE in adult rats leads to changes in the splicing pattern of sodium channel α-subunit mRNAs. Splice variants associated with neonatal development, designated IIN and IIIN, increased in their levels of expression following an increase in kainic acid-induced seizures [13]. These increases occur in the dentate gyrus and CA1 regions of the hippocampus, which are directly associated with this type of seizure [13]. It has been proposed that the role of alternative splicing in response to cellular activity may be important in drug and/or disease treatment design [54]. For example, possible treatment or diagnostic applications for breast cancer have been suggested using the $nNa_v1.5$ splice variant [55]. This splice variant results in alterations on the D1:S3 region of the protein, and this splice form is upregulated along with the progression of breast cancer and may potentially activate other cellular responses to induce metastasis [55]. If this relationship proves to be reliable, then increases in the levels of $nNa_v1.5$ splice forms may be used to test for the presence of metastic breast cancer. Such potential advantages further demonstrate the importance of uncovering the signaling pathways that allow alternative splicing to be involved in cellular responses.

Alternative splicing in other ion channels

Alternative splicing is not only utilized by sodium channel genes but is common for most channel genes, presumably to increase functional divergence and alter channel kinetics. For example, N-type $Ca_v2.2$ calcium channel splice variants undergo mutually exclusive exon splicing. The difference in exons leads to a splice variant with exon 37a, which has a longer open channel state than the splice variant that includes exon 37b [56]. Such kinetic changes may affect the amount of calcium that enters the cell following cellular excitation, which may lead to prolonged excitation. Changes in tissue localization due to alternative splicing have also been observed in other ion channels such as BK-type calcium-activated potassium channels, which are encoded by the slowpoke (Slo) gene. These splice variants are expressed in a tissue-specific manner to a degree that some exons are completely excluded from a specific tissue type, and the absence of an exon may be used for diagnostic purposes to identify specific tissues [57]. N-type calcium channels also demonstrate activity-dependent regulation of alternative splicing. For example, N-type channel splice variants containing a particular exon (e37a) are downregulated in response to neuropathic pain [58]. Such widespread alternative splicing in numerous ion channel types suggests that this phenomenon plays an important role in the regulation of cellular output and plasticity.

Post-translational regulation of ion channel function via phosphorylation

Protein phosphorylation constitutes one of the major molecular mechanisms influencing properties of voltage-gated channels, and therefore is a key player in the plasticity of neuronal function. Yet due to the multitude of potential phosphorylation sites in these channels and the relative difficulty of manipulating distinct states of phosphorylation, we are only beginning to understand the critical role this ubiquitous process plays in the regulation of ion channel function. The VGSCs are no exception to the rule that phosphorylation is a major player in influencing their function. This section of the review focuses on a few examples of the role kinases play in altering VGSC function and the resulting functional implications.

VGSC phosphorylation via PKA and PKC

Early biochemical experiments of purified brain VGSCs showed that the channels' α-subunits are substrates for phosphorylation by cAMP-dependant protein kinase (PKA) [59]. Moreover, phosphorylation of VGSCs in synaptosomes occurs rapidly following activation of PKA or PKC [60, 61]. Through cell culture of intact brain neurons, PKA induced-phosphorylation was found to be restricted to five consensus sites in the cytoplasmic linker to domains I and II of the VGSC [62–64]. The first evidence for modulation of VGSC function by PKA came from experiments on cloned VGSCs expressed in non-neuronal cell types. PKA phosphorylation in rat brain VGSCs expressed in *Xenopus* oocytes and Chinese hamster ovary (CHO) cells reversibly reduced peak current amplitudes in single-channel recordings but

did not affect the voltage-dependent activation or inactivation [65, 66]. Activation of D1-like dopamine receptors by its neurotransmitter in cultured rat hippocampal neurons reduced peak Na^+ current through VGSCs by PKA activation, suggesting that modulation of Na^+ currents play an important role in the inhibitory effects of dopamine on the hippocampus [67]. Recent evidence also suggests that PKA phosphorylation of VGSCs in hippocampal pyramidal cells requires PKA to be specifically anchored to the channel by A-kinase anchoring protein 15 (AKAP15), ensuring that the appropriate target is phosphorylated [68]. The functional significance of reducing peak currents in VGSCs has remained largely unknown, but recent evidence suggests a possible role for phosphorylation in altering inactivation kinetics (see below).

More recently, the influence of protein kinase C (PKC) on VGSC phosphorylation has been investigated. PKC phosphorylation sites are not only found in the domain I–II linker of VGSCs but are also located in the domain III–IV linker [69]. Like PKA, activation of PKC also reduces Na^+ currents of VGSC expressed in *Xenopus* oocytes and in neurons of the rat brain [70, 71]. Moreover, similar reductions in Na^+ currents have been seen in VGSC in various cultured rat brain neurons, including embryonic, hippocampal, and cortical neurons following activation of PKC by muscarinic acetylcholine receptor stimulation [72, 73]. Contrary to its effects on hippocampal and cortical neuron cultures, PKC activation increases Na^+ currents of tetrodotoxin resistant (TTX-R) VGSCs in a dose-dependent manner in cultured DRG neurons, as well as increases the rates of activation and inactivation of these channels [74, 75]. The functional effects of this phosphorylation result in increased Na^+ currents, presumably by increasing VGSC availability, as well as altering channel kinetics by changing activation and inactivation rates.

PKA and PKC do not have mutually exclusive effects on sodium channels, and may even have synergistic effects. For example PKC and PKA modulation converge on VGSC currents in rat brain neurons [66, 69, 76, 77]. PKC activation enhances PKA modulation of Na^+ currents at holding potential of -110 mV in cultured embryonic neurons and transfected CHO cells [66, 76]. Conversely, only a small effect of PKA on Na^+ current is observed without PKC activation at -110 mV holding potential [76, 77]. In the absence of PKC enhancement, significant reduction in Na^+ current due to PKA activation occurs at a holding potential of -75 to -80 mV as previously discussed [67, 77]. Mutagenesis experiments reveal that PKC-mediated enhancement of PKA requires PKC phosphorylation at two serine residues, one at the domain I–II linker and the other at the domain III–IV linker [69]. While the actual mechanism of synergistic effects of these two different kinases are yet to be determined, one possibility is that PKC induces a conformational change in the linker regions that reveals previously unavailable sites of PKA phosphorylation.

Effects of phosphorylation on biophysical properties of VGSC and neuronal output

Recent work suggests that phosphorylation of VGSCs can alter the activity of neurons due to a change in channel availability (see Fig. 3). Normally VGSCs that open in response to depolarization inactivate within milliseconds due to blockage of the inner mouth of the pore by the domain III–IV linker [78]. However, during prolonged depolarization a small number of channels enter a slow inactivation state, also referred to as a bursting mode, in which the channels can contribute to a small-sustained Na^+ current. This slow inactivation state is distinct from the normal fast inactivation [79–81], and in this state channels may be unavailable for periods lasting up to seconds, as they do not open in response to subsequent membrane depolarization. This can have profound effects on the output of cells. For example, the current elicited during slow inactivation is capable of delaying the repolarization of action potentials in cardiac cells, which can lead to cardiac arrhythmia [82, 83]. Furthermore, it has also been suggested that slow inactivation can render channels unavailable during repetitive firing of action potentials in neurons as they remain in this state for a period of seconds [84]. Mutations that impair slow inactivation can cause periodic paralysis of skeletal muscle and arrhythmias of the heart [85, 86], and various point mutations in different regions of VGSC have been shown to have small effects on slow inactivation [79, 80, 87–90]. However, no mutations have yet been described that substantially block slow inactivation, leaving the molecular mechanism largely unknown.

A comparison of slow inactivation with PKA/PKC neuromodulation in cortical neurons and transfected cells showed a close correlation between the two processes and suggests that PKA and PKC may enhance the slow inactivation of VGSCs [84]. Furthermore, activation of PKC with 5-HT receptor agonists results in increased thresholds for action potentials and shortened bursts of action potentials, primarily reflecting a change in VGSC availability [84, 91]. More recently, mutagenesis experiments further implicate phosphorylation in the mechanism modulating slow inactivation [92]. Previous work demonstrated that mutating residues of the S6 segments in bacterial sodium channels blocked slow inactivation

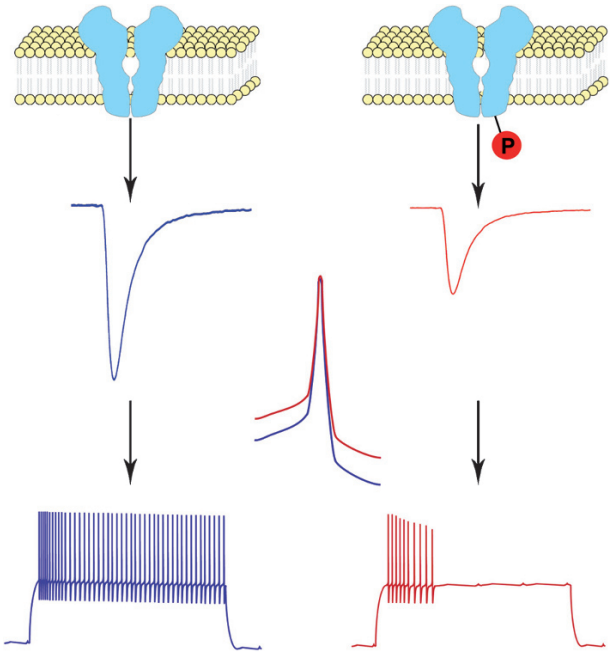

Figure 3. VGSC phosphorylation alters kinetics of an inward current and leads to spike failure. The same VGSC carries an inward current (left) that is altered when the channel is phosphorylated (right). Phosphorylation results in a reduction in inward current (middle panel). Furthermore, phosphorylation (e.g. via neuromodulation) also can induce a state of slow channel inactivation [84]. These alterations in channel properties and kinetics result in a depolarizing shift in the action potential threshold (inset) as well as a decrease in the ability of the neuron to fire during prolonged depolarization (bottom panel) [84].

[93]. Mutating highly conserved asparagine residues to alanines in the S6 segments in rat VGSCs increases slow inactivation and increases modulation by PKA/PKC activation [92]. Conversely, mutating the asparaginine residues to aspartates impairs slow inactivation and prevents modulation [92]. Taken together, these results suggest that modulation of the VGSCs by PKA/PKC enhance the intrinsic slow inactivation gating of the channels. In addition, modulation by PKA/PKC activation was more effective when the membrane was held at a more depolarized potential and when the neuronal activity was prolonged, correlating with the slow inactivation model [92]. These new findings provide a framework for better understanding the earlier data of alterations in VGSC current via phosphorylation of ion channels. The induction or enhancement of slow inactivation by phosphorylation of VGSCs can lead to alterations in threshold and in sustaining repetitive firing in cortical neurons. This modulation is on the timescale of seconds, endowing neurons with a form of short-term cellular plasticity which can affect synaptic integration as well as dendritic electrogenesis.

Another example of phosphorylation affecting neuronal output is revealed in the mechanisms of hyperalgesia in primary sensory neurons as a result of injury. Hyperalgesia is associated with abnormal bursting activity in primary sensory neurons involved in nociception [94, 95]. Heightened excitability of sensory neurons occurs within moments of an inflammatory stimulus [96]. This hyperexcitability is correlated with modulation of VGSC channel activity as PKA and PKC activation in cultured dorsal root ganglia (DRG) sensory neurons results in dose-dependant current increases in tetrodotoxin-resistant (TTX-R) VGSCs [74, 75, 97]. Furthermore, the rates of activation and inactivation are increased in these channels as well as a hyperpolarizing shift in the voltage dependence of activation [74, 75, 97]. Altering the kinetics of activation and inactivation of TTX-R channels, as well as lowering the threshold for activation, may allow channels to open and close more quickly during repetitive firing of action potentials. The end result of these changes may cause neurons to become hyperexcitable, firing repetitive action potentials more readily in response to a noxious stimulus.

Alteration of biophysical properties via phosphorylation is a common mechanism of regulating voltage-gated channel function

While the above discussion indicates that phosphorylation of VGSC can affect neuronal excitability, phosphorylation of voltage-gated K^+ (K_v) channels also can have important effects on neuronal function. Both PKA and PKC activation causes a reduction in overall fast-transient A-type K^+ current amplitude in dendrites of CA1 pyramidal neurons of the hippocampus [98]. A-type K^+ current underlies the attenuation of back-propagating action potentials (b-AP) in dendrites [99], so a reduction in this current leads to an increase in b-AP amplitude. B-APs spread passively to dendrites when the axon hillock generates an action potential [100] and are important for regulating the frequency of action potentials generated in the soma [100]. Phosphorylating A-type channels therefore indirectly regulate the frequency of action potentials generated by a neuron by reducing A-type K^+ currents, which has implications for signal processing of the neuron.

Phosphorylation of K_v channels can also regulate localization and trafficking of the channels within neurons. Mutation of multiple phosphorylation sites within the C-terminal domain of homomeric $K_v1.2$ channels and heteromeric $K_v1.2/1.4$ channels results in suppressed cell surface expression [101], suggesting that the phosphorylation state of these channels may affect their intracellular trafficking. $K_v1.2$ channels are capable of forming many different channel types, including low-threshold, sustained, or delayed recti-

fier K_v channels when coassembled with K_v1 α-subunits [102]. Moreover, gene knockout of $K_v1.2$ in mice enhances seizure susceptibility and results in death by postnatal day 17 on average [103], indicating that the regulation of $K_v1.2$ activity in neurons is important for normal brain function.

Finally, nociceptive sensitivity is also capable of being modulated through Kv channel phosphorylation. Activation of extracellular signal-regulated kinase (ERK) through metabotropic glutamate receptor (mGluR) activation results in nociceptive behaviors in mice [104]. $K_v4.2$ channels contain an ERK phosphorylation as suggested through mutational analysis [105]. Furthermore, ERK-dependent forms of hypersensitivity and ERK-mediated modulation in dorsal horn neurons are absent in $K_v4.2$ knockout mice [105]. More recently, mGluR5 activation was shown to modulate nociceptive information and ERK was found to be the downstream effector [106]. ERK activation then results in the reduction of $K_v4.2$-mediated A-type K^+ current, which is a determinant of neuronal excitability and underlies hypersensitivity of dorsal horn neurons after tissue injury [106].

Although a great deal of work has been done to generate an understanding of the impact of phosphorylation on ion channel function, we are only beginning to appreciate the diverse and important roles that this form of post-translational modification plays. Further work in elucidating other components involved in phosphorylation pathways, as well as generating even better animal models to study the effects of phosphorylation on ion channels and thus neuronal output will play a key role in understanding neuronal plasticity.

Subcellular membrane localization of ion channel proteins

The initial sections of this review have focused on the individual ion channel types that are responsible for electrical signaling in excitable cells. However, in addition to the type, abundance, and modification of the channel proteins themselves, another key feature required for generation of appropriate neuronal output is the precise distribution of ion channels along the plasma membrane of the cell. This ion channel localization is not necessarily a static property of a cell, but rather a dynamic process contributing yet another layer of complexity to neural signaling. We will address a classic and one of the best-understood examples of ion channel redistribution within a cell, the clustering of sodium channels into developing nodes of Ranvier during myelination, as a model for processes affecting ion channel localization.

Change in sodium channel expression and localization with myelination

Axons of the peripheral nervous system are unmyelinated at birth [107, 108]. Myelination begins postnatal day 1 (P1), and is complete around P14 within peripheral nerves (PNS, [108]). Myelination profoundly affects the underlying axon changing gene expression and protein localization organizing the axon into a series of domains referred to as internodes (regions of myelin) and nodes of Ranvier (regions devoid of myelin), which differ in protein content (for a more detailed review see [107]). Prior to myelination, peripheral [108] and central (CNS, [109–111]) nerves express the 1.2 α-subunit isoform of the VGSC ($Na_v1.2$; see Fig. 4). This expression pattern is maintained in CNS neurons through the myelination process [110]. However, recent evidence indicates that neurons of the PNS begin expressing low levels of $Na_v1.6$ at the earliest stages of myelination with the predominant isoform still being $Na_v1.2$ [112]. Once myelination is complete, mature nodes of peripheral axons predominantly express $Na_v1.6$ [108, 112]. Within the CNS, $Na_v1.6$ localizes to most nodes, but there remains a subset of axons that continue to express $Na_v1.2$ along with $Na_v1.8$ [109, 110]. Interestingly, in the hypomyelinated mouse *shiverer*, oligodendrocytes form aberrant junctions with the axon [113]. Axons of these mice have increased sodium channel densities [114, 115], but analysis of *shiverer* axons detected increased staining for $Na_v1.2$ in hypomyelinated regions of axons [110], suggesting that the developmental transition between $Na_v1.2$ and $Na_v1.6$ requires the precise interaction of myelinating cells with their CNS axons. While it is clear that clustering of sodium channels within nodes (see below) significantly increases the rate of action potentials (saltatory conduction), it is not clear how the developmental transition from $Na_v1.2$ to $Na_v1.6$ contributes to saltatory conduction, especially given that not all myelinated axons in the CNS undergo this transition. Prior to myelination, $Na_v1.2$ channels are localized diffusely throughout the axonal plasma membrane in both PNS [108, 112, 116, 117] and CNS [118–120]. However, once myelination is complete, not only has there been a transition from $Na_v1.2$ to $Na_v1.6$ in the majority of axons [108–110], these sodium channels are clustered within the node in both the PNS [108–110, 112, 116] and CNS [118, 119]. At birth, Schwann cells and PNS axons have obtained a 1:1 ratio [121] that will be maintained throughout adulthood, but at this early time, $Na_v1.2$ clusters are not observable [108]. As early as P1, $Na_v1.2$ clusters [108], and to a lesser extent $Na_v1.6$ clusters [112], are observable at the edges of the expanding Schwann cells. At P3 in the PNS and P10 in the CNS, these clusters appear as a

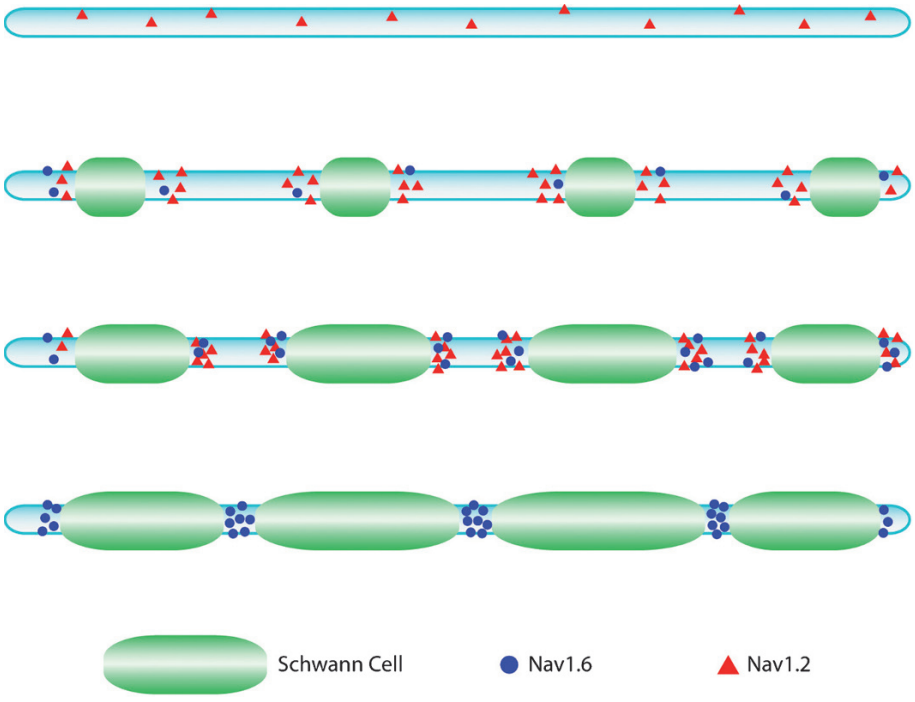

Figure 4. An example of VGSC localization in axons of the developing peripheral nervous system. During the development of the PNS, unmyelinated axons (top) contain diffusely localized $Na_v1.2$ VGSCs (red triangles). As Schwann cells begin to wrap and myelinate the axon, a progression of VGSC clusters made up largely of $Na_v1.2$ occurs at the edges of expanding Schwann cells. These channels become more tightly clustered as the nodes of Ranvier form, and ultimately transition from predominantly containing $Na_v1.2$ to almost exclusively being made up of $Na_v1.6$ (blue circles) clusters in the mature nodes.

single broad cluster in between two myelinating cells referred to as 'broad clusters' [108]. Broad clusters seem to fuse into two-channel aggregates resulting in little to no channel staining in between these newly formed clusters, forming 'presumptive nodes' in which sodium channels are no longer spread over a wide gap but instead form highly focal clusters in the PNS [108, 112, 122] and CNS [110].

While the physiological significance of transitioning to $Na_v1.6$ expression is unclear, one potential reason for maintaining $Na_v1.2$ expression during CNS myelination is the differential control of clustering between these two α-subunit isoforms [111]. An as yet unidentified peptide released from oligodendrocytes induces clustering of sodium channels [111, 118]. However, only the $Na_v1.2$ isoform responds to the oligodendrocyte-derived clustering factor; the $Na_v1.6$ isoform fails to cluster in the presence of soluble or contact-mediated signals [111, 118].

Mechanistically, it is unclear whether clustering requires lateral movement of existing sodium channels along the axonal plasma membrane, differential targeting of newly synthesized sodium channels or both. Within the CNS, inhibition of both protein trafficking and protein synthesis in cultured retinal ganglion cells prevents sodium channel clustering, yet neither treatment affects maintenance of existing clusters [111]. Additionally, oligodendrocyte-conditioned medium did not increase the cell surface sodium channel density [111]. $Na_v1.2$ clusters are associated with the edge of the elongating myelinating cell in both the PNS [108, 122, 123] and CNS [110, 119]. To maintain this localization throughout myelination would seem to require lateral diffusion of existing $Na_v1.2$ channels along the cell surface. Interestingly, the majority (approximately 80%) of the expressed $Na_v1.2$ channels in unmyelinated axons do not readily diffuse within the axonal plasma membrane [117]. Taken together, these data suggest that existing, cell surface localized sodium channels cluster due to the expression and trafficking of a non-sodium channel protein. While the identity of this protein is unknown, it is interesting to speculate that the identity of the newly expressed protein may be a novel component of the axonal cytoskeleton or a protein the alters the affinity of sodium channels with the existing cytoskeletal network.

Mechanisms of channel localization: Is contact with myelinating cells required to form sodium channel clusters?

Throughout myelination, the different clustering stages are associated with myelinating cells that are in contact with the axon. However, it is not clear that contact between myelinating cells and their axons is required. Within the PNS, loss of Schwann cell contact with the axon seems to preclude sodium channel clustering [108, 116, 117, 122, 124, 125]. However, within the CNS, evidence suggests that oligodendrocyte-axon contact is not required for the induction of $Na_v1.2$ clustering [111, 118, 126, 127]. In fact, mature oligodendrocytes may release a soluble protein factor

that is sufficient to induce clustering of $Na_v1.2$ channels [111, 118]. Treating oligodendrocyte-conditioned medium with heat or trypsin reduced its ability to induce sodium channel clustering [118]. Furthermore, in several hypomyelinated mouse models, node formation and sodium channel clustering is still evident *in vivo* [118, 119, 126, 127]. Contact-independent induction of clustering is an inherent property of the oligodendrocyte. Culturing CNS neurons with PNS myelinating cells (Schwann cells) only induced clustering if the Schwann cells were in contact with the axons, whereas culturing PNS axons with CNS myelinating cells (oligodendrocytes) resulted in clusters even when the oligodendrocytes were not in contact with the axon [111]. These data suggest that Schwann cells and oligodendrocytes utilize a common mechanism to induce sodium channel clustering with the difference being that the soluble oligodendrocyte clustering factor [118] may be a surface protein in Schwann cells.

The degree of node formation and sodium channel clustering also may be dependent on the maturation level obtained by oligodendrocytes. Cultured oligodendrocytes induce clustering only after they reach 1 week postdifferentiation [111]. In the hypomyelinated mouse *jimpy*, oligodendrocytes spontaneously degenerate within the first postnatal weeks [128–130]. However, there is no alteration in the number of sodium channel clusters observed in these mice relative to wild-type mice [126, 127]. Maturation state of oligodendrocytes is not the only consideration. Oligodendrocytes in the hypomyelinated mouse *shiverer* can mature much further than oligodendrocytes in *jimpy*, yet sodium channel clustering is reduced [119]. This is not to imply that the CNS does not require oligodendrocytes. As noted above, the transition from $Na_v1.2$ to $Na_v1.6$ requires precise interaction between oligodendrocytes and axons [110]. Moreover, in the hypomyelinated mouse *shiverer*, sodium channel clusters are reduced in number and appear in aberrant locations [119]. The few nodes that do form in the *shiverer* mouse display aberrant morphology [110], as judged by the precise localization of nodal proteins (see [107]). Additionally, once mature nodes have formed, contact of oligodendrocytes with the axon is required for maintenance of sodium channel clusters [131].

Functional implications for altered channel localization: sodium channels in demyelinated axons

Ultimately the importance of ion channel localization is the effect that channel density in specific areas of membrane has on the functional output of the neurons. In the case of VGSC localization, altered channel localization is often the result of disruption of the interactions between ion channels and myelin as a result of demyelination. While there are many conditions that can lead to the loss of myelin, multiple sclerosis (MS) is the most common neurological disorder affecting young adults. MS can follow one of four different clinical courses, all of which result in loss or damage to CNS myelin that can result in impairment of neuronal conduction [132]. Sodium channel expression and localization has been analyzed in postmortem patient tissue and in the experimental autoimmune encephalomyelitis (EAE) animal model of MS. In both MS [133] and EAE [134, 135], $Na_v1.2$ and $Na_v1.6$ expression is upregulated in demyelinated regions of axons. The diffuse localization of $Na_v1.2$ and $Na_v1.6$ in demyelinated axons in MS is similar to the pattern observed in pre-myelinated nerve fibers in both the PNS and CNS. These data suggest that re-expression and diffuse localization of $Na_v1.2$ may support action potentials within demyelinated axonal segments, thus allowing patients to regain lost functions during periods of remission in the absence of remyelination [132], although it is likely that conduction within the demyelinated section of nerves would be substantially slower than in myelinated regions of the same axon. This would likely affect the characteristics of the action potential when moving from nerve sections with differing states of myelination. Interestingly, the presence of $Na_v1.6$ in demyelinated axons may be associated with increased vulnerability to axonal injury, which is also observed in MS [132]. In mouse models in which myelin fails to form, $Na_v1.2$ channels expression is maintained [110, 136]. Moreover, dysmyelinated axons are less susceptible to calcium-mediated cell death [137]. $Na_v1.6$ channels produce larger persistent currents than $Na_v1.2$ channels allowing more sodium into the axon [138]. In the EAE mouse model of MS, $Na_v1.6$ channels co-localize with the sodium-calcium exchanger [134]. Sustained influxes of sodium through $Na_v1.6$ channels can alter intracellular sodium concentration, resulting in the sodium-calcium exchanger reversing its operation [139], which leads to increased intracellular calcium levels [140]. Maintenance of $Na_v1.6$ channels may, therefore, predispose MS axons to calcium-mediated injury [132].

Precise ion channel localization is a universal factor in cellular function

Myelination results in the precise re-localization of a once diffuse population of sodium channels. However, sodium channels are not the only channels affected by myelination, and ion channel localization is important to the mediation of more biological processes than just saltatory conduction. Potassium channel localization is also affected by myelination [107], and can be

dramatically altered as a result of seizure events [141]. Moreover, defective calcium channel clustering in growth cones is observed in a mouse model of the childhood disease spinal muscular atrophy [142]. Further demonstrating the ubiquitous need for correct channel localization, chemotaxis in the sea urchin, *A. punctulata* relies upon calcium influx into sperm upon the binding of the chemotatic molecule resact [143], which may activate the calcium channel through the production of cGMP [144]. Therefore, beyond the expression and production of the appropriate channel, proper function of the ion channel in the cell also is dependent on precise localization. Further research is needed in this critical area of neuronal signaling to develop a complete understanding of how functional neural output is achieved.

Conclusions

The concepts presented in this paper barely begin to scratch the surface of the complexity and abundance of mechanisms involved in regulating both the properties of voltage-gated channel proteins and their subsequent effect on neuronal output. Furthermore, the discrete sections imply that the mechanisms of expression, splicing, phosphorylation, and localization are mutually exclusive portions of a complex regulatory process. Yet recent work demonstrates this is certainly not the case. For example, the trafficking and localization of the voltage-gated potassium channel $K_v1.2$ are largely regulated by a specific cluster of C-terminal phosphorylation sites, and not by signals encoded in its primary structure [101]. Splice variants of $Na_v1.3$ result in channel proteins with differing consensus phosphorylation sites, which presumably impact the properties of these cells [47]. Finally, myelination is known to influence not only VGSC localization in the axon but also gene expression of VGSC subtypes in the neurons [107]. These studies demonstrate that we are only beginning to understand the complex, multi-modal processes that ultimately result in functional voltage-gated ion channels.

The extraordinary diversity and complexity of ion channel genes, proteins, and mechanisms for altering the function of these key players in neuronal output should not be surprising when one considers that ion channels represent one of the most ancient forms of communication between a cell and its external environment. Indeed, it is possible to trace the origins of eukaryotic voltage-gated channels to proteins of highly similar primary structure found in prokaryotes [145]. Voltage-gated channels abound in organisms as diverse as paramecia, algae, jellyfish, and of course arthropods and mammals [2]. Therefore, evolution has been honing the properties of these proteins for hundreds of millions of years. The end result is the plethora of channel genes, multiple mechanisms to influence the resulting proteins, and finally complex neuronal output and behavior. Further study will only continue to add to an already rich knowledge of these ubiquitous and critical proteins.

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