Regulation of voltage-gated ion channels in excitable cells by the ubiquitin ligases Nedd4 and Nedd4-2

Daria Bongiorno, Friderike Schuetz, Philip Poronnik and David J. Adams

1Health Innovations Research Institute and School of Medical Sciences; RMIT University; Melbourne, Victoria; 2School of Biomedical Sciences; The University of Queensland; Brisbane, Queensland Australia

†Current address: Health Innovations Research Institute; RMIT University; Melbourne, Victoria Australia

‡These authors contributed equally to this work.

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Abbreviations: CAM, cell adhesion molecule; CBS, cystathionine-β-synthase; CIC, chloride channel; DUBs, deubiquitinating enzymes; ER, endoplasmic reticulum; IGF-1R, insulin-growth factor 1 receptor; Kv, voltage-gated potassium channel; M-channel, KCNQ2/3 and KCNQ3/5 channel; Na+, voltage-gated sodium channel; Nedd4, neural precursor cell-expressed developmentally downregulated gene 4; Nedd4-2, neural precursor cell-expressed developmentally downregulated gene 4-2; Ndfips, Nedd4 family interacting protein; PY, proline-rich; SGK, serum- and glucocorticoid kinase; UPS, ubiquitin proteasome system

The electrical excitability of neurons is mediated primarily by voltage-gated ion channels, particularly voltage-gated Na⁺ (NaVs), K⁺ (Kvs) and Cl⁻ (CIC) channels. Cells regulate their electrical excitability by controlling not only the activity, but also the number of individual ion channels in the plasma membrane. There exist several mechanisms for regulating levels of voltage-gated ion channels: transcription and translation, retention and export from the endoplasmic reticulum as well as insertion and retrieval from the plasma membrane. Alterations in voltage-gated ion channel activity, composition and distribution can contribute to the pathophysiology of epilepsy, hypertension, neuropathic and inflammatory pain. One mechanism for retrieval is ubiquitination. Here specific ubiquitin ligases bind to membrane proteins to modulate and regulate their cellular fate. In this review, we focus on Nedd4 and Nedd4-2 ubiquitin ligases and the mechanisms by which they regulate voltage-gated ion channels and describe a novel paradigm on the mechanisms that underpin aberrant ion channel function in neurological disorders.

Introduction

Voltage-gated ion channels are located along the axon and at the synapse of neurons where they initiate and propagate action potentials, set the resting membrane potential and control neurotransmitter release thereby playing a primary role in regulating neuronal excitability. These transmembrane proteins are designated voltage-gated channels because their current-voltage relationships exhibit a high sensitivity to the membrane potential such that under physiological conditions they allow the inward movement of Na⁺ and the outward movement of K⁺ upon membrane depolarization in nerve cells. Thus characterizing the molecular mechanisms that regulate either single channel activity or total number of channels at the cell surface level is fundamental to any understanding of the complexity of neuronal signaling in health and disease.

The main classes of voltage-gated ion channels that regulate neuronal excitability are the voltage-gated Na⁺ (NaVs), K⁺ (Kvs) and Cl⁻ (CIC) channels. Their structures provide important clues as to the various possible mechanisms of regulation. Voltage-gated ion channels are composed of several transmembrane spanning domains arranged around a central ion conducting pore and a C-terminal domain that contains recognition motifs that are important for the protein-protein interactions that ultimately regulate cell surface expression. Despite considerable research into their structure, function, pharmacology and signaling pathways, many of the actual protein-protein interactions that govern the regulation of these channels remains in many cases to be fully elucidated in detail. In this review, we will focus on the relatively recent data showing that cell surface levels of voltage-gated ion channels can be regulated by ubiquitin ligase mediated trafficking pathways. Given that abnormal activity or regulation of voltage-gated ion channels is thought to contribute substantially to the pathology of various neurological diseases such as epilepsy and neuropathic pain, this may represent a new paradigm to define changes in voltage-gated ion channel function in disease.

Voltage-gated ion channel structure and function. NaVs are composed of highly glycosylated α-subunits that form
formed similarly, but comprise four separate polypeptide subunits (Fig. 1A and B).

To date, nine genes of the ClC family have been identified in mammals. ClCs differ in subcellular localization and the ionic pore, as well as auxiliary β-subunits. To date ten mammalian α-subunits, NaV1.1-1.9 and the atypical NaV1.6, have been identified. Each α-subunit is composed of a single polypeptide consisting of 4 homologous domains (I–IV). Each homologous domain is made up of six transmembrane spanning helices (S1-S6) with S4 being a positively charged voltage-sensing helix (Fig. 1A and B). The α-subunits form the functional channel, however, the β-subunits are required for normal kinetics and voltage-dependent gating.6,7 KVs are formed similarly, but comprise four separate polypeptide subunits (Fig. 1A and B).

To date, nine genes of the ClC family have been identified in mammals. ClC proteins co-assemble to form dimers that contain 18 transmembrane spanning domains (A to R) and cytosolic domains containing two large cystathionine-β-synthase (CBS) motifs (Fig. 1C). ClCs are trafficked to the cell surface, with their CBS domain particularly important for channel function and expression.6 ClCs differ in subcellular localization and
tissue distribution. For example, CIC1 and CIC2 are located in skeletal muscle and neurons respectively, with the former establishing and restoring the resting membrane potential of skeletal muscle and the latter regulating neuroexcitability in the brain. Although the precise mechanisms involved in CIC function remain poorly understood, their participation in maintaining neuronal excitability is clear.

**Trafficking of Voltage-Gated Ion Channels**

Maintaining the appropriate levels of voltage-gated ion channels in the plasma membrane underpins normal patterns of excitability. It is now recognized that ion channels and other membrane proteins exist, not only in functionally discrete membrane domains, but also in dynamic intracellular pools from which they can be rapidly mobilized to the membrane. Thus the density of ion channels at the plasma membrane represents a dynamic equilibrium between their insertion, retention and retrieval at the membrane. Thus the density of ion channels at the plasma membrane represents a dynamic equilibrium between their insertion, retention and retrieval at the plasma membrane. A classic example of localization of Na⁺ channels is the clustering of Na⁺ channels into the developing Nodes of Ranvier during myelination. At the molecular level, protein-protein interactions facilitate and direct Na⁺ channel traffic. For example, the protein annexin II light chain (pI1) binds to the N-terminus of Na⁺,1.8 to enhance its movement to the plasma membrane. Na⁺,1.5 mutation associated with Brugada syndrome that blocks ankyrin-G binding, also disrupts Na⁺,1.5 surface expression in cardiomyocytes.

Ankyrin G, a scaffolding protein, has been shown to bind to the pore-forming α-subunit of some Na⁺s in both neuronal and non-neuronal cells, implying a potential role for ankyrin G in the trafficking of Na⁺s to and from the membrane surface. An example supporting such a tenet comes from a recent study demonstrating that a Na⁺,1.5 mutation prevents binding of ankyrin G to Na⁺,1.5, in turn contributing to the altered expression of Na⁺,1.5 in cardiomyocytes that ultimately leads to fatal cardiac arrhythmias in patients with Brugada syndrome. This is further supported by findings that a reduction in ankyrin G leads to decreased Na⁺,1.5 channel expression and current density in cardiomyocytes. In neuronal cells following peripheral nerve trauma, both ankyrin G, Na⁺,1.7 and Na⁺,1.8 are re-distributed in the unmyelinated axon in an attempt to restore electrical excitability and thus normal neuronal function. However, in patients with painful neuromas, ankyrin G, Na⁺,1.7 and Na⁺,1.8 are further upregulated suggesting that ankyrin G is responsible for the axonal hyperexcitability that occurs via an overexpression of axonal Na⁺ channels which contributes to the pain condition.

Na⁺s have also been identified to contain an IQ motif in their C-terminus allowing for Ca²⁺ dependent regulation. This is supported by recent findings that Na⁺s are regulated by calmodulin in isolated guinea-pig ventricular myocytes. In skeletal muscle, the calmodulin binding site on Na⁺,1.4 channels is also able to facilitate channel trafficking and thus expression at the cell surface. Furthermore, the activation of protein kinase A or contactin, a glycosyl-phosphatidylinositol-anchored cell adhesion molecule (CAM) protein, promotes trafficking and increases the expression of cardiac Na⁺ in the plasma membrane, whereas protein kinase Cα can induce Ca²⁺-induced endocytosis of Na⁺.

Trafficking of K⁺ channels is also subject to tight regulation, with the C-terminal and pore domains being particularly important. Bioactive molecules such as dendrotoxin K, calnexin, calmodulin, Rab-GTPase, cyclic AMP, as well as dynem are able to affect cell surface expression and trafficking. In addition, auxiliary subunits such as KCNE or β subunits, can also modulate K⁺ channel localization and trafficking. Other mechanisms include the depletion of phosphatidylinositol 4,5-biphosphate (PIP₂) which results in the suppression of KCNO/K⁺,7 channels from the membrane. In addition, the TCC2 domain in the C-terminus of KCNO channels is crucial for efficient transport of heteromeric channels to the plasma membrane.

**Ubiquitination of Target Proteins: Role in Trafficking of Voltage-Gated Ion Channels**

The above examples highlight the importance and complex trafficking mechanisms that mediate the plasma membrane levels of voltage-gated ion channels. The insertion of ion channels in the membrane must be balanced by their removal. In this context, one other mechanism discovered relatively recently, ubiquitination, is also gaining in interest as a potential regulator of voltage gated ion channels under both normal conditions and pathological states. Ubiquitin is a highly conserved 76 amino acid polypeptide that serves as a tag for the internalization and degradation of membrane proteins thereby playing a major role in protein degradation. Ubiquitination of proteins requires three sequential enzymatic steps. First, a ubiquitin activating enzyme E1, activates the ubiquitin; second, the activated ubiquitin is transferred to a cysteine residue on an E2 ubiquitin conjugating enzyme and third, the E2 enzyme associates with an E3 ligase that transfers the ubiquitin to lysine residues on the target protein. Although the family of E3 ligases requires E1 and E2 enzymes, it is the E3 ligases that bind to recognition motifs in the target protein and is therefore the enzyme that confers target protein specificity.

Ubiquitination itself is a complex process and the level and type of ubiquitination dictates the fate of the target protein. The ubiquitin molecule itself contains seven lysine residues each of which can be involved in the formation of chains of ubiquitin molecules. A target protein can be either monoubiquitinated (one ubiquitin on one lysine residue), multi-monoubiquitinated (multiple lysine residues by one ubiquitin molecule) or polyubiquitinated (binding of many ubiquitin molecules). Formation of ubiquitin chains on K48 typically leads to the targeting and degradation of the protein in the proteasome. In contrast, ubiquitination on K63 directs the protein for trafficking or lysosomal degradation. These two distinct pathways highlight both the
Table 1. Tissue distribution and PY-motifs of voltage-gated ion channels (VGIC)

| VGIC | Distribution | PY motifs in the C-terminal domain of VGIC |
|------|--------------|-------------------------------------------|
| NaV1.1 | CNS/PNS | S T A C C P P S Y D R V T K |
| NaV1.2 | CNS | P S T T S P P S Y D S V T K |
| NaV1.3 | CNS | S S T T S P P S Y D S V T K |
| NaV1.5 | CNS/heart | S S T S F P P S Y D S V T R |
| NaV1.6 | CNS/PNS | P S T A S L P S Y D S V T K |
| NaV1.7 | PNS | A S T I S P P S Y D S V T K |
| NaV1.8 | CNS | S A T S F P P S Y D S V T R |
| K,1.3 | Lymphocytes | I D I V A I I I P Y F I T L G |
| K,7.1 | Heart/kidney | L P S N T L P T Y E Q L T V |
| K,7.2 | CNS/PNS | K E P E P A P Y P Y H S P E D |
| K,7.3 | CNS/PNS | E T G P P E P P Y S F H Q V |

CNS, central nervous system; PNS, peripheral nervous system. PY motif marked in red. Conserved amino acid sequences marked in grey.

specificity and diversity of ubiquitin mediated protein regulation contributing to many cellular functions, such as the activation and silencing of transcription, signal transduction, apoptosis, immune and inflammatory response, cell cycle, receptor mediated endocytosis and various metabolic pathways such as autophagy. It is therefore not surprising that mutations in E1, E2 and E3 ligases are also associated with neurological disorders. For example, in familial juvenile Parkinson’s disease (PD), a defect in the E3 ligase Parkin results in the impairment of toxic protein aggregates removal resulting in neuronal cell death and thus neurodegeneration. In Amyotrophic Lateral Sclerosis (ALS), the E3 ubiquitin ligase Dorfin, has recently been shown to be critical for mutant superoxide dismutase enzyme (SOD) degradation, in both animal models and human patients. Importantly, the underlying pathology of these disorders results in part to abnormally aggregated protein, that may result due to disruption of E3 ubiquitin ligase mediated degradation.

In addition to the above-mentioned neurological disorders, defects in E3 ligase activity may also have specific consequences on levels of membrane transport of proteins. For example, conditions such as epilepsy and pain are due to abnormal regulation of sodium channels by the ubiquitin proteasome system (UPS). A deeper understanding of the molecular mechanisms involved in the UPS mediated degradation of proteins and ion channels may provide novel insights into mechanisms that may alleviate these debilitating diseases. It is important to note, that although there is ample evidence implicating the UPS in neurological diseases, the molecular identity of the ligases and associated proteins remains largely unknown.

**Nedd4 and Nedd4-2: E3 Ubiquitin Ligases**

Nedd4 and the closely related protein Nedd4-2 (neuronal precursor cell-expressed developmentally downregulated protein 4 and 4-2) are E3 ubiquitin ligases. Nedd4 was first identified as a mouse gene that is highly expressed in early embryonic development of the CNS. Nedd4 is expressed widely, whereas Nedd4-2 is expressed in the heart, kidney, brain, lung and liver, and both are highly conserved in the eukaryotic cell. Nedd4 and Nedd4-2 consist of a calcium/lipid binding (CaLB/C2) domain, three or four tryptophan-rich (WW) domains and an E6-AP-C-terminal (HECT) domain that are similar to human papilloma virus (HPV) oncoprotein E6-associated protein (E6-AP). The C2 domain, a calcium-binding domain, is approximately 120 amino acids in length and believed to regulate the function of proteins by translocating them to phospholipid membranes. The WW domains consist of 35–40 amino acids and two conserved tryptophan residues that are 21 amino acids apart. These domains consist of a hydrophobic core surrounded by β-sheets and interact with the proline-rich (PY) motifs of the substrate protein. The most common proline-rich sequence recognized by the WW domains is the PPxY motif (Table 1). However, WW domains are also known to bind alternative or atypical motifs for example Nedd4-2 was reported to bind to the amino acid sequence LPxY (Table 1). Therefore, the Nedd4-like proteins are able to recognize a number of different motifs via their WW domains other than the typical PPxY motif, and these phosphoserine and phosphothreonine modules could be important for Nedd4 ligase interactions.

The HECT domain which is approximately 350 residues in length, transfers ubiquitin from the conserved cysteine residue, located on the carboxyl end of the HECT domain to a lysine residue of the target protein. The initial physiological role for the Nedd4 family of proteins was identified in Liddle’s syndrome. The extreme hypertension associated with Liddle’s syndrome results from increased Na+ reabsorption by the kidneys due to increased cell surface expression of the epithelial Na+ channel (ENaC). ENaC consists of three subunits (α, β and γ) each of which contain C-terminal PY motifs. Although both Nedd4 and Nedd4-2 can bind to and ubiquitinate all three ENaC subunits, Nedd4-2 is the true physiological mediator. In the normal kidney, ubiquitination by Nedd4-2 plays a key role in controlling the levels of ENaC at the luminal surface of the kidney tubules by targeting their degradation and thereby regulating Na+ reabsorption. In Liddle’s syndrome, mutations in the C-terminal region of ENaC prevent this interaction with Nedd4-2. This prevents the degradation of ENaC which in turn leads to an accumulation of ENaC at the

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cell surface with the increased Na\textsuperscript{+} reabsorption accounting for the extreme hypertension.\textsuperscript{55,56}

The number of target proteins for Nedd4 and Nedd4-2 continues to grow and it is clear that Nedd4 and Nedd4-2 are two distinct proteins with separate physiological effectors. This is highlighted in a recent proteomic screen demonstrating that although they are closely related and share a similar structure, they have distinct substrates and thus may have distinct physiological functions.\textsuperscript{37}

The current binding substrates for Nedd4 include insulin-growth factor 1 receptor (IGF-1R),\textsuperscript{39} epidermal growth factor receptor (EGFR),\textsuperscript{59,60} vascular endothelial growth factor receptor 2 (VEGFR2),\textsuperscript{41} as well as the tumor suppressor gene, PTEN (phosphatase and tensin homolog deleted on chromosome 10).\textsuperscript{38,62,63} Nedd4-2 on the other hand has been shown to regulate dopamine active transporter (DAT),\textsuperscript{14,67} the glutamate transporter, excitatory amino acid transporters 1 and 2 (EAAT1/2),\textsuperscript{66,67} serum- and glucocorticoid kinase 1 (SGK1), the transforming growth factor-\beta receptor (TGF\textbeta R),\textsuperscript{68} and neurotrophin receptor, TrkA.\textsuperscript{69} In addition, Nedd4-2 has been shown to regulate a number of ion channels, such as the chloride channels CIC-2 and CIC-5, as well as the Tewey family of chloride channels,\textsuperscript{70} voltage-gated potassium channels KCNQ1,\textsuperscript{71} 2/3, 3/5,\textsuperscript{72} 4.3,\textsuperscript{74} in addition to a variety of voltage-gated sodium channels.\textsuperscript{75-77}

**Nedd4 and Nedd4-2 Regulation of Voltage-Gated Ion Channels**

More recently, it has been recognized that Nedd4 and Nedd4-2 can regulate voltage-gated ion channels. Voltage-gated Na\textsuperscript{+} channels have an important role in the generation and propagation of action potentials in electrically excitable cells. Seven voltage-gated sodium channels (Na\textsubscript{1.1}, 1.2, 1.3, 1.5, 1.6, 1.7 and 1.8) contain the PY motif in their C-terminus that is recognized by Nedd4 and Nedd4-2 (Table 1). Both Nedd4 and Nedd4-2 have been shown to directly interact with voltage-gated sodium channels, however, Nedd4 appears to be less effective in regulating channel activity than Nedd4-2. Nedd4-2 has been shown to downregulate Na\textsuperscript{+} current densities of Na\textsubscript{1.2}, 1.3, 1.7 and 1.8 via the PY motif\textsuperscript{73-76} with disruption in the PY motif of Na\textsubscript{1.8} resulting in no change in density of this channel.\textsuperscript{73} Understanding the regulation of these channels by Nedd4 and Nedd4-2 may be useful, for example, in development of novel therapeutics. This is particularly important in nociceptive neurons, where the upregulation of Na\textsubscript{1.8} can lead to a reduction in pain thresholds.\textsuperscript{13,78} Furthermore, Na\textsubscript{1.5} has recently been shown to be upregulated in astrocytes within multiple sclerosis lesions.\textsuperscript{79} Nedd4 and Nedd4-2 are also able to regulate Na\textsubscript{1.5} in cardiac muscle\textsuperscript{77} where it is involved in the generation and propagation of cardiac action potentials. Mutations in the subunits of Na\textsubscript{1.5} that alter its cell surface expression, are linked to drug-acquired long QT syndromes (cardiac arrhythmias), Brugada syndrome, Lenegre-Lev syndrome, conductive disorders and infant death syndrome.\textsuperscript{80,81} Since the C-terminus of Na\textsubscript{1.5} contains a PY motif that is recognized by Nedd4-2, it appears that it is a negative regulator of this cardiac Na\textsubscript{1.5} channel.\textsuperscript{82} Furthermore, studies indicate that Nedd4 and Nedd4-2 can regulate the cell surface expression of this channel without changing the biophysical properties or activity.\textsuperscript{77}

Cl\textsuperscript{-} channels are also regulated by ubiquitination. CIC-2 channels have been reported to be a target of Nedd4-2.\textsuperscript{83} In Xenopus oocytes, co-expression of Nedd4-2 and CIC-2 leads to a significant downregulation of chloride channel current and diminished CIC-2 cell surface expression. Cell surface expression of CIC-5 channels in Xenopus oocytes are also reduced by Nedd4-2.\textsuperscript{84,85}

Voltage-gated K\textsuperscript{+} channels also play a major role in regulating membrane potential and modulate electrical excitability in neurons and muscles by determining action potential firing, duration and after-hyperpolarization.\textsuperscript{86} These channels are important in all cell types and influence the regulation of cell volume, synaptic transmission, as well as muscle contraction.\textsuperscript{87-91} Recent studies have shown that only Nedd4-2 is able to ubiquitinate individual KCNQ2 and three subunits, whereas the downregulation of K\textsuperscript{+} current amplitudes is mediated by KCNQ heteromers (Fig. 2) without a change in cell surface levels.\textsuperscript{72} Interestingly, although the C-terminus is required the PY motif does not appear to be involved. This was supported by Glutathione S-transferase fusion protein (GST) pull-down and co-immunoprecipitation studies showing an interaction between KCNQ3 C-terminus and Nedd4-2 even when the tyrosine (Y) is mutated to an alanine (A) at position 698. This suggests that Nedd4-2 may bind to a different, yet unidentified intracellular motif on target proteins. Previous studies have shown sequences that are similar but not identical, such as LPTY can bind to Nedd4-2.\textsuperscript{92} The binding motif of KCNQ2 and 3 subunits that is recognized by Nedd4-2 remains to be elucidated. Similarly, for K\textsubscript{1.3} channels that does not contain a PY motif, Nedd4-2 is able to reduce peak K\textsuperscript{+} current amplitude by 50% in Xenopus oocytes.\textsuperscript{73} Therefore, Nedd4-2 may be able to regulate these channels through chaperones and accessory proteins such as KChAP, which do contain PY motifs.\textsuperscript{93} This is relevant, as mutations in KCNQ2 and 3, which are responsible for the muscarine-sensitive K\textsuperscript{+} current (M-current) in central neurons, are associated with benign familial neonatal convulsions.\textsuperscript{94,95} and these channels are also able to interact with accessory subunits. In addition, altered K\textsubscript{v} channels with KChIP4 are implicated in Alzheimer's disease.\textsuperscript{96} Therefore, the regulation of channel expression at the membrane by these accessory subunits may be a novel mechanism for the involvement of Nedd4 and Nedd4-2.

KCNQ1 channels have an atypical PY-motif in their C-terminal (L/P/PxYxxΦ). Nedd4-2 reduces the total amount of KCNQ1 protein by labeling this channel for degradation via this motif.\textsuperscript{71} This mechanism is likely to be important for the regulation of cell surface density of KCNQ1 channels in cardiomyocytes and other cell types. Furthermore, it highlights that Nedd4 and Nedd4-2 channel regulation is complex, and goes beyond interactions between WW domains of these two ligases and PY motifs on the target protein and channels. Supporting this view, it is known that the regulation of IGF-1R, is mediated by growth factor receptor-binding protein 10 (Grb10α). IGF-1R itself does not contain a PY motif, but its negative regulator Grb10α does, therefore Nedd4 regulates IGF-1R indirectly. Other examples of indirect regulations involve SGK1, which is a substrate of Nedd4-2 (see below).
Other Proteins Involved in Regulating Ubiquitin-Mediated Trafficking of Voltage-Gated Ion Channels

Serum- and glucocorticoid kinase (SGK). SGK1 was originally identified in a rat mammary tumor cell line, where mRNA levels increase dramatically when cells are exposed to serum, glucocorticoids or both. SGK1 and its related isoforms SGK2 and SGK3 share 80% amino acid sequence homology of their catalytic domains. They are members of the “AGC” subfamily, which include protein kinase A, G and C, and SKG1 has a similar catalytic domain to protein kinase B. SGK1 expression is induced by a broad spectrum of stimuli including IGF-1, aldosterone, neuronal injury, neuroexcitotoxicity, psychophysiological stress and memory consolidation. SGK kinases are widely expressed in mammals and are highly conserved in eukaryotic cells from yeast to human. SKG1 is established as a kinase acting as a cell survival molecule.

As mentioned above, IGF-1 can activate SGK1, and is thought to do so via by phosphoinositide-dependent kinase 1 (PDK1)-induced phosphorylation. PDK1 is a direct downstream target of phosphatidylinositol 3-kinase (PI 3-kinases), and thus IGF-1 mediated signaling. In humans, SGK1 consists of a catalytic domain with the C-terminus containing a PY motif that is able to interact with Nedd4-2. The phosphorylation of Nedd4-2 by SGK1 leads to its inactivation, and the interaction between these two molecules is thought to contribute to the regulation of many proteins and ion channels such as ENaC, EAAT1 and 2, as well as the voltage-gated ion channels KV1.3. For example, studies in Xenopus oocytes show that SGK1 is able to increase ENaC cell-surface expression and thus may be the functional mediator for aldosterone-dependent regulation of ENaC as well as negatively regulating Nedd4-2. An increase in cell surface expression of EAAT1/2 transporters by IGF-1 through activation of PDK1 has also been shown to occur via its downstream target SGK1. The regulation of both ENaC and EAAT1/2 is thought to occur as a consequence of SGK phosphorylation of Nedd4-2, and subsequent decrease in the interaction of Nedd4-2 and its target channels and transporters. CIC2 channels have also been reported to be a target of Nedd4-2 with SGK (1-3).
enhanced CIC2 activity and cell surface expression decreased by Nedd4-2. In Xenopus oocytes, co-expression of Nedd4-2 and CIC2 leads to a significant downregulation of chloride channel current and enhanced CIC2 cell surface expression due to the ability of SGK to inactivate Nedd4-2. Finally, SGK1 has been suggested to also influence Nedd4-2 activity of K, although K does not contain a PY motif, suggestive of Nedd4-2 acting via an alternative accessory protein such as KChAP. Furthermore, when it comes to K, cell surface regulation, SGK1 is unable to abolish even at high concentrations the full extent of Nedd4-2 effect, and conversely, high concentrations of Nedd4-2 could not significantly inhibit the effect of SGK1 effect.

In summary, to date, several different mechanisms are known to regulate plasma membrane channel activity by SGK1 (Fig. 3): (i) direct phosphorylation of the target protein at a consensus sequence (RxRxxS/T) for SGK1, (ii) phosphorylation of the ubiquitin ligase Nedd4-2 and therefore inhibiting its interaction with the target channel protein, and (iii) interaction with trafficking molecules including NHERF2. As illustrated here, SGK1 modifies a variety of cellular functions by modulating the activity of membrane proteins including voltage-gated ion channels. A recent review further discusses the potential role of SGK1 in neuronal function. We can now begin to examine other voltage-gated ion channels that do not contain the typical PY motif as possible candidates of ubiquitination and thus degradation by Nedd4 and Nedd4-2 and ultimately the ubiquitin proteasome system.

Nedd4 binding proteins. It is also important to note that we are now also uncovering indirect mechanisms by which the two ligases, Nedd4 and Nedd4-2, are able to regulate their substrates. For example, Nedd4 (-/-) mice show a significant growth abnormality due to aberrant IGF-1 signalling. However, this phenotype arises largely due to Nedd4 ubiquitinating the adaptor Grb10, which normally stabilizes the IGF-1 receptor. We know that other intermediate ‘adaptor’ proteins, such as Ndfip1 (Nedd4 family interacting protein) may be crucial for Nedd4 and Nedd4-2 regulation of their substrates. Recent studies suggest that Ndfip1 may be responsible for the termination of Nedd4-mediated ubiquitination by recruiting Nedd4 and Nedd4-2 into exosomes. Furthermore, Ndfip1 has been shown to recruit Nedd4-2 to ubiquitinate DMT1 (divalent metal transporter 1). This is an important constitutive regulatory mechanism to maintain appropriate levels of metal ion transport critical for normal neuronal function.

Another important class of interacting proteins that has emerged to have significant impact in Nedd4-2 mediated substrate interactions is the highly conserved family of 14-3-3 proteins. These proteins can bind to a multitude of substrates through a RXRXXS motif, where pS is phosphorylated serine residue. Nedd4-2 was initially shown in Xenopus oocytes to be a substrate for 14-3-3, through its SGK1 binding site (RxRxxS/T). Subsequently it was found that aldosterone increases the expression of 14-3-3 in renal collecting duct cells. In these cells 14-3-3 binds to phosphorylated Nedd4-2, and...
prevents it from interacting with ENaC. This in turn leads to an increase in ENaC cell surface density and Na+ reabsorption.117 These findings clearly demonstrate sequestration of phosphorylated Nedd4-2 by 14-3-3 is an important regulatory mechanism to be considered in other cellular systems.

**Deubiquitinating proteins.** It is also important to note that the process of ubiquitination itself is under regulatory control and one mechanism to achieve this is deubiquitinating enzymes (DUBs). These enzymes serve important functions such as acting as proofreaders to remove ubiquitin from inappropriately tagged substrates, rapidly de-ubiquitinating degraded substrates in the proteasome to maintain an available pool of ubiquitin and lastly, processing ubiquitin precursors into conjugation-competent ubiquitin proteins.118 This highlights not only the complexity of ubiquitin-processing ubiquitin precursors into conjugation-competent ubiquitin but also the specificity and tight controls that they exert. Currently, the identity of the DUBs that are involved in the Nedd4 family of ligases remains to be determined. Abnormalities in both E3 ligases and DUBs have been implicated in neurodegenerative disease,119,120 as well as cancers.120

**Conclusion**

Numerous studies provide convincing evidence that PY motifs in the C-terminus of voltage-gated ion channels act as binding sites for the Nedd4 and Nedd4-2 WW domains. However, we have also highlighted the importance of indirect mechanisms of ion channel cell surface expression modulated by Nedd4 and Nedd4-2. These interactions ultimately determine ubiquitination of these channels, and thus subsequent internalization and degradation providing the control mechanism for levels at the plasma membrane. Importantly, Nedd4 and Nedd4-2 regulation of voltage-gated ion channels does not alter voltage-dependent gating properties but only the channel density. Mutations in voltage-gated ion channels lead to diseases such as epilepsy, migraine, Brugada syndrome, long QT1 syndrome and neuropathic pain.121,122 All of these voltage-gated ion channels disorders share in common a change in neuronal excitability that underpin the pathology.

In light of these findings, further investigations are required to elucidate whether Nedd4 and Nedd4-2 may also control cell surface expression of other voltage-gated ion channels. In particular, ion channels that do not contain the typical PY motif should be examined, as there are numerous examples of intermediate and tight controls that they exert. Currently, the identity of the DUBs that are involved in the Nedd4 family of ligases remains to be determined. Abnormalities in both E3 ligases and DUBs have been implicated in neurodegenerative disease, as well as cancers.120

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**References**

1. Carterell WA, Goldin AL, Waxman SG. International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. Pharmacol Rev 2005; 57:397-409.

2. Duran C, Thompson CH, Xiao Q, Hartzell HC. Chloride channels: often enigmatic, rarely predictable. Annu Rev Physiol 2010; 72:95-121.

3. Gutman GA, Chaody KG, Grimmer S, Ladunski M, McKinnon D, Parodi LA, et al. International Union of Pharmacology. LIII. Nomenclature and molecular relationships of voltage-gated potassium channels. Pharmacol Rev 2005; 57:473-508.

4. Thomas EA, Reid CA, Berkovic SF, Petruo S. Prediction by modeling that epilepsy may be caused by very small functional changes in ion channels. Arch Neurol 2009; 66:1225-32.

5. Dib-Hajj SD, Black JA, Waxman SG. Voltage-gated sodium channels: therapeutic targets for pain. Pain Med 2009; 10:1260-9.

6. Isom L, De Jongh K, Paton D, Reber B, Offord J, Charbonneau H, et al. Primary structure and functional expression of the β1 subunit of the rat brain sodium channel. Science 1992; 256:839-42.

7. Isom LL, Ragdale DS, De Jongh KS, Westenbroek RE, Reber B, Scheuer T, Carterell WA. Structure and function of the β2 subunit of brain sodium channels, a transmembrane glycoprotein with a canonical motif. Cell 1995; 85:433-42.

8. Carr G, Simmons N, Sayer J. A role for CBS domain 2 in trafficking of chloride channel CLC-5. Biochem Biophys Res Commun 2003; 310:600-5.

9. Sile S, Vanoye CG, George AL Jr. Molecular physiology of renal CIC chloride channels/transporters. Curr Opin Nephrol Hypertens 2006; 15:511-6.

10. Sik S, Smith RL, Freund TF. Distribution of chloride channel-2-immunoreactive neuronal and astrocytic processes in the hippocampus. Neuroscience 2000; 101:51-65.

11. Schulz DJ, Temporal S, Barry DM, García ME. Mechanisms of voltage-gated ion channel regulation: from gene expression to localization. Cell Mol Life Sci 2008; 65:2215-31.

12. Okuse K, Malik-Hall M, Baker M, Poon W, Kong H, Chao M, Wood J. Annexin II light chain regulates sensory neuron-specific sodium channel expression. Nature 2002; 417:653-6.

13. Shao D, Okuse K, Djamgoz MBA. Protein-protein interactions involving voltage-gated sodium channels: post-translational regulation, intracellular trafficking and functional expression. Int J Biochem Cell Biol 2009; 41:1471-81.

14. Mohler P, Rivolta I, Napolitano C, LeMaille G, Lamberr S, Pirisi S, Bennet V, Na, 1.5 Elt63K mutation causing Brugada syndrome blocks binding to ankyrin-G and expression of Na, 1.5 on the surface of cardiomyocytes. Proc Natl Acad Sci USA 2004; 101:17533-8.

15. Lowe J, Pylegin O, Bhasin N, Hund T, Boyden P, Shihata E, et al. Voltage-gated Na, channel targeting in the heart requires an ankyrin-G-dependent cellular pathway. J Cell Biol 2008; 180:173-86.

16. Kretschmer T, Nguyen DH, Beurmann RW, Happel LT, England JD, Lai RL, Klimek DG. Painful human neuromas: a potential role for a structural transmembrane protein, ankyrin G. J Neurosci 2002; 97:1424-31.

17. Mori M, Konno T, Ozawa T, Murata M, Imoto K, Nagayama K. Novel interaction of the voltage-dependent sodium channel (VDSC) with calmodulin: does VDSC acquire calmodulin-mediated Ca2+ sensitivity? Biochemistry 2000; 39:1316-23.

18. Aiba T, Hesker GG, Liu T, Carlisle R, Ville-Abrille MC, O’Rourke B, et al. Na+ channel regulation by Ca2+/calmodulin and Ca2+/calmodulin-dependent protein kinase II in guinea-pig ventricular myocytes. Cardiovasc Res 2010; 85:454-63.

19. Yuill KH, Smirnov SV. Calcium-dependent regulation of voltage-gated sodium channels in cardiac myocytes: just the beginning? Cardiovasc Res 2010; 85:411-2.

20. Bowers S, Desclées D, DiSilvestre D, Tian Y, Halperin VL, Tomarelli GF. Calmodulin regulation of Na,1.4 current: role of binding to the carboxyl terminus. J Gen Physiol 2008; 131:197-209.

21. Hallal H, Yang Z, Wivianathan PC, Fukuda K, Shen W, Wang DW, et al. Quantification of protein kinase A-mediated trafficking of cardiac sodium channels in living cells. Cardiovasc Res 2006; 72:250-61.

22. Cusdin FS, Clare JL, Jackson AP. Trafficking and cellular distribution of voltage-gated sodium channels. Traffic 2008; 9:17-26.

23. McEwen DP, Schumacher SM, Li Q, Benson MD, Högstedt-Lühr JM, Van Genderen KM, Martens JR. Rab-GTPase-dependent endocytic recycling of Kc, 1.5 in atrial myocytes. J Biol Chem 2007; 282:29612-20.

24. Vacher H, Mohapatra DP, Misonou H, Trimmer JS. Regulation of K,1 channel trafficking by the mamba snake neurotoxin dendrotoxin K. FASEB J 2007; 21:986-14.

25. Manganesi LN, Trimmer JS. Calnexin regulates mammalian K,1 channel trafficking. Biochem Biophys Res Commun 2004; 322:577-84.

26. Erexberria A, Aivar P, Rodríguez-Alfaro JA, Alaimo A, Villace P, Gomez-Posada JC, et al. Calmodulin regulates the trafficking of KCNQ2 potassium channels. FASEB J 2008; 22:1135-43.

27. Choi WS, Khurana A, Mathur R, Wivianathan V, Steele DF, Fedida D, K, 1.5 surface expression is modulated by retrograde trafficking of newly endocytosed channels by the dynein motor. Circ Res 2005; 97:363-71.

28. Connors EC, Balfi BA, Motelli AD. Homeostatic regulation of K,1.2 potassium channel trafficking by cyclic AMP. J Biol Chem 2008; 283:3445-53.

29. Bal M, Zhang J, Hernandez CC, Zaika O, Shapiro MS. Ca2+/calmodulin disrupts AKAP79/150 interactions with KCNQ (M-Type) K+ channel. Physiol Rev 2010; 90:755-96.
Kaneis L, 1997; 40:435-43.
H, Kinoshita M. Altered expression of sodium channel expression and the molecular patho-
H 1997; 40:435-43.
Ciechano Y, 1992; 185:1155-61.
Emson PC, Clare JJ. Changes in the mRNAs encod-
Pflügers Archiv 2006; 452:290-9.
Knaus, Bruce M, Skrynnikov N, Rotin D, Forman-
Kozarich J, van Bemmelen MX, Bruce MC, Jepsen T, Gavillet B, Apostolhs F, et al. Molecular determinants of voltage-gated sodium channel regulation by the 'Nedd4/Nedd4-like proteins.' Am J Physiol Cell Physiol 2005; 288:692-701.
Baker MD, Wood JN. Involvement of Na+ channels in pain pathways. Trends Pharmacol Sci 2001; 22:27-31.
Black JA, Newcombe J, Wismann SG. Astrocytes within multiple sclerosis lesions upregulate sodium channel Na+ 1.5. Brain 2010; 135:835-46.
Töibs-Hansen J, Winkel BG, Gruner M, Jepsen T. Inherited cardiac diseases caused by mutations in the Na+ 1.5 sodium channel. J Cardiovasc Electrophysiol 2010; 21:107-15.
Chen Q, Kirsch GE, Zhang D, Brugada R, Brugada J, Brugada P, et al. Genetic basis and molecular mecha-
nisms for idiopathic ventricular fibrillation. Nature 1998; 392:293-6.
Brehl H, Kamynta E, Horiberger JD, Staub O. Regulation of the cardiac voltage-gated Na+ channel (H1) by the ubiquitin-protein ligase Nedd4. FEBS Lett 2000; 466:377-80.
Palmada M, Dieter M, Boehmer C, Walleger S, Lang F. Serum and glucocorticoid inducible kinases functionally regulate CIC-2 channels. Biochem Biophys Res Commun 2004; 321:1001-6.
Hryciw DH, Ekberg J, Lee A, Lentsink IL, Kumar S, Guggino WB, et al. Nedd4-2 functionally interacts with CIC-5: involvement in constitutive acidic lumen endocytosis in proximal tubule cells. J Biol Chem 2004; 279:54996-5007.
Rieckheit G, Wartosch L, Schaffer S, Stobrawa SM, Novarino G, Weiler S, Jentsch TJ. Role of CIC-5 in renal endocytosis is unique among CIC exchangers and does not require PY motifs for ubiquitination. J Biol Chem 2010; 285:17599-603.
Pongs O. Regulation of excitability by potassium chan-
nels. Results Probl Cell Differ 2008; 44:145-61.
Brown AM. KChAP/K Vb1.2 interactions and their effects on cardiac KV channel expression. Am J Physiol 2010; 261:49-61.

Watabiki T, Takasugi N, et al. Molecular cloning and characterization of CALP/KChIP4, a novel EF-hand protein kinase subunit. J Biol Chem 2002; 277:14965-75.

Groenewegen WA, van Kempen MJA, et al. Functional synaptic reorganization. J Physiol 2008; 586:3405-23.

Chen Y, Zeng R, Shen B, Jia J, Wang Y. Neuronal trans-synaptic stimulation stimulates the phosphorylation of K V1.4 channel protein at Ser229 through protein kinase A1. J Neurochem 2010; 27:16973-8.

Naray-Fejes-Toth A, Canessa C, Cleaveland ES, Aldrich G, Fejes-Toth G, sgl is an aldosterone-induced kinase in the renal collecting duct. Effects on epithelial Na+ channels. J Biol Chem 1999; 274:16973-8.

Imaiuzumi K, Tsuda M, Watanaka A, Tsuchiya M, Takagi T. Differential expression of sgl mRNA, a member of the Ser/Thr protein kinase gene family, in rat brain after CNS injury. Brain Res Mol Brain Res 1994; 26:189-96.

Nishida Y, Nagata T, Takahashi Y, Sugahara-Kobayashi M, Murata A, Asai S. Alteration of serum/glucocorticoid regulated kinase-1 (sgk-1) gene expression in rat hippocampus after transient global ischemia. Brain Res Mol Brain Res 2004; 123:121-5.

Hollister RD, Page KJ, Hyman BT. Distribution of the messenger RNA for the extracellularly regulated kinases 1, 2 and 3 in rat brain: effects of exogenous hippocampal lesions. Neuroscience 1997; 79:11-11.

Murata S, Yoshida T, Lim CR, Sugino M, Kogure M, Ohnuki T, et al. Psychophysiologically stress-regulated gene expression in mice. FEBS Lett 2005; 579:2137-42.

Tsai KJ, Chen SK, Ma YL, Hsu WL, Lee EH, sgl, a primary glucocorticoid-induced gene, facilitates memory consolidation of spatial learning in rats. Proc Natl Acad Sci USA 2002; 99:9990-5.

Waldheger S, Barth P, Raber G, Lang F. Cloning and characterization of a putative human serine/threonine protein kinase transcriptionally modified during aisononic and isotonic alterations of cell volume. Proc Natl Acad Sci USA 1997; 94:4440-5.

Shanmugam I, Cheng G, Terranova P, Thresher J, Thomas C, Li B. Serum/glucocorticoid-induced protein kinase-1 facilitates androgen receptor-dependent cell survival. Cell Death Differ 2007; 14:2085-94.

Kumari S, Liu X, Nguyen T, Zhang X, D’Mello SR. Distinct phosphorylation patterns underlie Akt activation by different survival factors in neurons. Mol Brain Res 2001; 96:157-62.

dela Rosa DA, Zhang P, Naray-Fejes-Tóth A, Fejes-Tóth G, Canessa CM. The serum and glucocorticoid kinase sgl increases the abundance of epithelial sodium channels in the plasma membrane of Xenopus oocytes. J Biol Chem 1999; 274:37834-9.

Loffing J, Zecovic M, Feralle E, Kaissling B, Asher C, Rosier BC, et al. Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: possible role of SGK. Am J Physiol Renal Physiol 2001; 280:675-82.

Lang F, Strutz-Seebohm N, Seebohm G, Lang U. Significance of SGK1 in the regulation of neuronal function. J Physiol 2010; 580:1688-97.

Hollister RD, Page KJ, Hyman BT. Distribution of the messenger RNA for the extracellularly regulated kinases 1, 2 and 3 in rat brain: effects of exogenous hippocampal lesions. Neuroscience 1997; 79:11-11.

Murata S, Yoshida T, Lim CR, Sugino M, Kogure M, Ohnuki T, et al. Psychophysiologically stress-regulated gene expression in mice. FEBS Lett 2005; 579:2137-42.

Tsai KJ, Chen SK, Ma YL, Hsu WL, Lee EH, sgl, a primary glucocorticoid-induced gene, facilitates memory consolidation of spatial learning in rats. Proc Natl Acad Sci USA 2002; 99:9990-5.

Waldheger S, Barth P, Raber G, Lang F. Cloning and characterization of a putative human serine/threonine protein kinase transcriptionally modified during aisononic and isotonic alterations of cell volume. Proc Natl Acad Sci USA 1997; 94:4440-5.

Shanmugam I, Cheng G, Terranova P, Thresher J, Thomas C, Li B. Serum/glucocorticoid-induced protein kinase-1 facilitates androgen receptor-dependent cell survival. Cell Death Differ 2007; 14:2085-94.

Kumari S, Liu X, Nguyen T, Zhang X, D’Mello SR. Distinct phosphorylation patterns underlie Akt activation by different survival factors in neurons. Mol Brain Res 2001; 96:157-62.

dela Rosa DA, Zhang P, Naray-Fejes-Tóth A, Fejes-Tóth G, Canessa CM. The serum and glucocorticoid kinase sgl increases the abundance of epithelial sodium channels in the plasma membrane of Xenopus oocytes. J Biol Chem 1999; 274:37834-9.

Loffing J, Zecovic M, Feralle E, Kaissling B, Asher C, Rosier BC, et al. Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: possible role of SGK. Am J Physiol Renal Physiol 2001; 280:675-82.

Lang F, Strutz-Seebohm N, Seebohm G, Lang U. Significance of SGK1 in the regulation of neuronal function. J Physiol 2010; 580:1688-97.

Putz U, Howitt J, Lackovic J, Poor N, Kumar S, Silke J, Tan SS. Nedd4 family-interacting protein 1 (Ndfip1) is required for the exosomal secretion of Nedd4 family proteins. J Biol Chem 2008; 283:32621-7.

Howitt J, Putz U, Lackovic J, Poor A, Dunston L, Cheng H, et al. Divalent metal transporter 1 (DMT1) regulation by Ndfip1 prevents metal toxicity in human neurons. Proc Natl Acad Sci USA 2009; 106:15489-94.

Fu H, Subramanian RR, Masters SC. 14-3-3-Proteins: structure, function and regulation. Annu Rev Pharmacol Toxicol 2000; 40:417-47.

Nagaki K, Yamamura H, Shimada S, Saito T, Hisanaga Si, Taoka M, et al. 14-3-3 mediates phosphorylation-dependent inhibition of the interaction between the ubiquitin E3 ligase Nedd4-2 and epithelial Na+ channels. Biochemistry 2005; 44:673-46.

Ichimura T, Yamamura H, Sasamoto K, Tominaga Y, Taoka M, Kakiuchi K, et al. 14-3-3 proteins modulate the expression of epithelial Na⁺ channels by phosphorylation-dependent interaction with Nedd4-2 ubiquitin ligase. J Biol Chem 2005; 280:13187-94.

Bhalia Y, Dadide D, Li H, Pao AC, LaGrange LP, Wang J, et al. Serum- and glucocorticoid-regulated kinase 1 regulates ubiquitin ligase neural precursor cell-expressed, developmentally downregulated protein 4-2 by inducing interaction with 14-3-3. Mol Endocrinol 2005; 19:3073-84.

Li X, Peters KW, Butterworth MB, Frizzell RA. 14-3-3 isoforms are induced by aldosterone and participate in its regulation of epithelial sodium channels. J Biol Chem 2006; 281:16323-32.

Amerik AY, Hochstrasser M. Mechanism and function of deubiquitinating enzymes. Biochimica et Biophysica Acta 2004; 1695:189-207.

Sestia R, Wada K. The functions of UCH-L1 and its relation to neurodegenerative diseases. Neurochem Int 2007; 51:105-11.

Hussain S, Zhang Y, Galarly P. DUBs and cancer: The role of deubiquitinating enzymes as oncogenes, non-oncogenes and tumor suppressors. Cell Cycle 2009; 8:1688-97.

Carrell WA, Dib-Haj S, Meisler MH, Pietrobon D. Inherited neuronal ion channelopathies: new windows on complex neurological diseases. J Neurosci 2008; 28:11768-77.

Maljevic S, Würtke TV, Leche H. Nervous system K₇ disorders: breakdown of a subthreshold brake. J Physiol 2008; 586:1791-801.