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Regulation of Neuronal Voltage-gated Sodium Channels by the Ubiquitin-Protein Ligases Nedd4 and Nedd4-2*

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Nedd4 and Nedd4-2 are ubiquitin-protein ligases known to regulate a number of membrane proteins including receptors and ion transporters. Regulation of the epithelial Na^+ channel by Nedd4 and Nedd4-2 is mediated via interactions between the PY motifs of the epithelial sodium channel subunits and the Nedd4/Nedd4-2 WW domains. This example serves as a model for the regulation of other PY motif-containing ion channels by Nedd4 and Nedd4-2. We found that the carboxyl termini of the six voltage-gated Na^+ (Na_v) channels contain typical PY motifs (PP*X*Y), and a further Na_v contains a PY motif variant (LP*X*Y). Not only did we demonstrate by Far-Western analysis that Nedd4 and Nedd4-2 interact with the PY motif-containing Na_v channels, but we also showed that these channels have conserved WW domain binding specificity. We further showed that the carboxyl termini fusion proteins of one central nervous system and one peripheral nervous system-derived Na^+ channel ($Na_v1.2$ and $Na_v1.7$, respectively) are readily ubiquitinated by Nedd4-2. In *Xenopus* oocytes, Nedd4-2 strongly inhibited the activities of all three Na_vs ($Na_v1.2$, $Na_v1.7$, and $Na_v1.8$) tested. Interestingly, Nedd4 suppressed the activity of $Na_v1.2$ and $Na_v1.7$ but was a poor inhibitor of $Na_v1.8$. Our results provide evidence that Nedd4 and Nedd4-2 are likely to be key regulators of specific neuronal Na_v channels *in vivo*.

Voltage-gated sodium channels (Na_vs)[1] are essential for the generation and propagation of action potentials in electrically excitable cells. These channels mediate the influx of Na^+ ions in response to local depolarizing stimuli and thus play key roles in regulating excitation, secretion, and contraction (1). Na_v channel activity is crucial for the plasticity and development of the nervous system and the maintenance of excitability following nerve and tissue injury (1). Na_v channels are composed of a pore-forming α-subunit (~260 kDa) associated with one or more auxiliary β-subunits (~35 kDa) (1). The α-subunits are able to form functional channels, whereas the β-subunits modulate the biophysical properties of the α-subunit (2). Nine mammalian Na_v channel α-subunit genes have been characterized with distinct tissue-specific expression and electrophysiological properties (3). Based on expression profiles, individual Na_v channel genes can be divided into several different groups. $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$ are primarily found in the central nervous system; $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$ are predominant in the peripheral nervous system, and skeletal and cardiac muscle cells express $Na_v1.4$ and $Na_v1.5$, respectively (3). The expression profile of the Na_v channel types in excitable cells contributes to the specificity of their transduction and excitability properties (1). The importance of normal Na_v channel activity is evident from hereditary channel mutations that result in epilepsy, long QT syndrome, Brugada syndrome, and many other diseases (2).

Although it is known that trafficking and recycling is critical for the regulation of numerous ion channels, little is known about the regulation of Na_v channels by this mechanism. It is well established that members of the Nedd4-family of ubiquitin-protein ligases (4–6), Nedd4 and Nedd4-2 in particular, regulate the activity of the epithelial sodium channel (ENaC) in response to high intracellular Na^+ via ubiquitination and removal from the plasma membrane (7–13). The Nedd4 family of proteins is composed of an amino-terminal Ca^{2+}/phospholipid binding domain, a carboxyl-terminal catalytic HECT domain, and multiple WW domains (protein-protein interaction modules) (6). Specific WW domains of Nedd4 proteins interact with PY motifs in the carboxyl termini of ENaC subunits to facilitate channel ubiquitination and down-regulation (10, 13). In Liddle's syndrome, a hereditary form of hypertension, deletions/mutations in ENaC PY motifs preclude normal interactions with Nedd4 family WW domains, thereby resulting in elevated Na^+ channel activity (7, 8).

Interestingly, several Na_v channel family members have a highly conserved PY motif in the carboxyl termini of their α-subunits. There has, however, been only one report on the regulation of a Na_v channel (the cardiac channel $Na_v1.5$) by Nedd4 (14). This study, performed in *Xenopus* oocytes, showed that Nedd4 reduced Na^+ currents mediated by $Na_v1.5$, an effect that was not observed when the PY motif was mutated (14). However, a direct physical interaction of Nedd4 and $Na_v1.5$ was not demonstrated. To date, there are no reports of the regulation of neuronal Na_v channels by the Nedd4 family of ubiquitin-protein ligases. In the present study, we identify Na_v channel α-subunits that contain intracellular PY motifs and examine their ability to interact with Nedd4 and Nedd4-2. We show that all PY motifs containing Na_v channels can bind specific WW domains in Nedd4 and Nedd4-2. Furthermore, two representative Na_v channels were tested and

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[1] The abbreviations used are: Na_v(s), voltage-gated sodium channel(s); ENaC, epithelial sodium channel; PY motif, PP*X*Y sequence; GST, glutathione *S*-transferase; PVDF, polyvinylidene fluoride.

found to be ubiquitinated *in vitro* by Nedd4-2. When expressed in *Xenopus* oocytes with Na_v channel α-subunits, Nedd4 and Nedd4-2 were found to down-regulate the depolarization-activated Na^+ currents.

MATERIALS AND METHODS

Bacterial Expression Plasmids—The Na_v channel carboxyl-terminal region-glutathione *S*-transferase (GST) fusion constructs were generated as follows. The carboxyl termini were PCR-amplified from mouse brain cDNA ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$), mouse day 18 embryo cDNA ($Na_v1.5$ and $Na_v1.7$), and mouse dorsal root ganglion cDNA ($Na_v1.8$) (donated by A. Stokowski) and cloned into either the BamHI or BamHI/EcoRI sites of pGEX-2TK (Amersham Biosciences). The number of carboxyl-terminal residues present in the various Na_v-GST fusion proteins are as follows: $Na_v1.1$, 64; $Na_v1.2$, 66; $Na_v1.3$, 62; $Na_v1.5$, 72; $Na_v1.6$, 57; $Na_v1.7$, 64; and $Na_v1.8$, 81. A construct containing mouse Nedd4-2 (ΔC2,WW1,WW2) was generated by PCR amplification from mouse Nedd4-2 cDNA followed by cloning into the EcoRI site of pGEX-2TK. A catalytically inactive Cys mutant of Nedd4-2 (ΔC2,WW1,WW2) was produced by PCR mutagenesis. Constructs containing mouse Nedd4 and Nedd4-2 WW domains and carboxyl termini of α, β, and γENaC in pGEX-2TK have been described previously (10, 12, 13). The construct containing mouse Nedd4 (amino acids 52–777) in pGEX was a gift of J.-P. Jensen and A. M. Weissman (NCI, National Institutes of Health).

Production of GST Fusion Proteins—Overnight cultures of *Escherichia coli* BL21 star (DE3)pLysS (Invitrogen) harboring the Nedd4-2 (ΔC2,WW1,WW2) or Nedd4-2 (ΔC2,WW1,WW2) Cys mutant GST expression plasmids were diluted 1:25, grown to log phase at 22 °C, and then induced with 1 mM isopropyl β-D-thiogalactoside at 22 °C. All other GST fusion proteins were grown similarly but at 37 °C. Following induction, GST fusion proteins were purified as described previously (10, 12, 13). The protein concentrations were measured using a BCA kit (Pierce) and Coomassie Blue staining following separation by SDS-PAGE.

SDS-PAGE and Far-Western Analysis—^{32}P-labeled protein probes were produced by directly labeling the appropriate GST fusion protein using protein kinase A (New England Biolabs) as described previously (15). For Far-Western analysis, ~2 μg of each GST fusion protein was resolved by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (PerkinElmer Life Sciences). Membranes were blocked in Hyb75 and then hybridized with the appropriate ^{32}P-labeled protein probes for 4 h at 4 °C in Hyb75 as described (15). Membranes were washed four times in Hyb75 and visualized by autoradiography. Band intensities were analyzed semi-quantitatively using ImageQuant Version 5.2 software (Molecular Dynamics). Far-Western binding intensities were measured against background probe hybridization levels except for bands representing the wild type individual WW domains, which were measured relative to the nonspecific binding of the corresponding mutant WW domains. To normalize binding intensities within individual Far-Western blots, these values were divided by the intensity of corresponding Coomassie Blue-stained protein bands and expressed in arbitrary units.

In Vitro Ubiquitination Assays—Carboxyl-terminal $Na_v1.2$ and $Na_v1.7$ GST fusion proteins and GST alone were ^{32}P-labeled by protein kinase A as described above. ^{32}P-labeled proteins were incubated with 750 ng of purified Nedd4 (amino acids 52–777), GST, Nedd4-2 (ΔC2,WW1,WW2), or the Nedd4-2 (ΔC2,WW1,WW2) Cys mutant in the presence of rabbit E1 (150 ng) and UbcH5b (300 ng) (both from Boston Biochem Inc.) in 25 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, 500 μM dithiothreitol, and 500 ng/μl bovine ubiquitin (Sigma) for 2 h at 30 °C. 15-μl reactions were stopped by the addition of a reducing sample buffer containing SDS, resolved by SDS-PAGE, and visualized by autoradiography.

Electrophysiological Recording of Na_v Channel α-Subunit-mediated Currents in Xenopus Oocytes—Capped RNA transcripts encoding full-length α-subunits of human $Na_v1.8$ (a gift of R. Lewis, University of Queensland, and R. Drinkwater, Xenome Inc., Brisbane, Australia), the human $Na_v1.8$ PY mutant, rat $Na_v1.7$ (a gift of G. Mandel, State University of New York), rat $Na_v1.2$ (a gift of A. Goldin, University of California), Nedd4, and Nedd4-2 and the ligase-defective Cys mutants of these ligases were synthesized using a mMESSAGE mMachine *in vitro* transcription kit (Ambion). To disrupt the PY motif of $Na_v1.8$ (PPSY), Tyr-1921 was mutated to an Ala (Y1921A) using standard PCR-based, site-directed mutagenesis with *Pfu* Turbo polymerase (Stratagene). *Xenopus laevis* stage V-VI oocytes were removed and treated with collagenase (Sigma type I) for defolliculation. The oocytes were then injected with combinations of the cRNA of the Na_v channel (2 ng/cell) with the Nedd4 or Nedd4-2 cRNAs (10 ng/oocyte). The oocytes were incubated at 18 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 5 mM pyruvic acid, and 50 μg/ml gentamicin, pH 7.5) prior to recording. Three days after cRNA injection, whole cell Na^+ channel currents were recorded from oocytes using the two-electrode (virtual ground circuit) voltage clamp technique. Microelectrodes were filled with 3 M KCl and typically had resistances of 0.3–1.5 megohms. All recordings were made at room temperature (20–23 °C) using bath solution containing 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, and 20 mM Hepes, pH 7.5, with NaOH. During recording, oocytes were perfused continuously at a rate of ~1.5 ml/min. Using a GeneClamp 500B amplifier and pCLAMP 8 software (Axon Instruments Inc, Union City, CA), data were low pass filtered at 1 kHz, digitized at 10 kHz, and leak-subtracted on-line using a -P/6 protocol and analyzed off-line. Initially, inward Na^+ currents were generated by holding the cells at −70 mV and applying step depolarizations to membrane potentials from −50 mV to +50 mV. Inward Na^+ currents were evoked with 100-ms depolarizing pulses at 10-s intervals to +20 mV ($Na_v1.8$) and 0 mV ($Na_v1.7$ and $Na_v1.2$) from a holding potential of −70 mV.

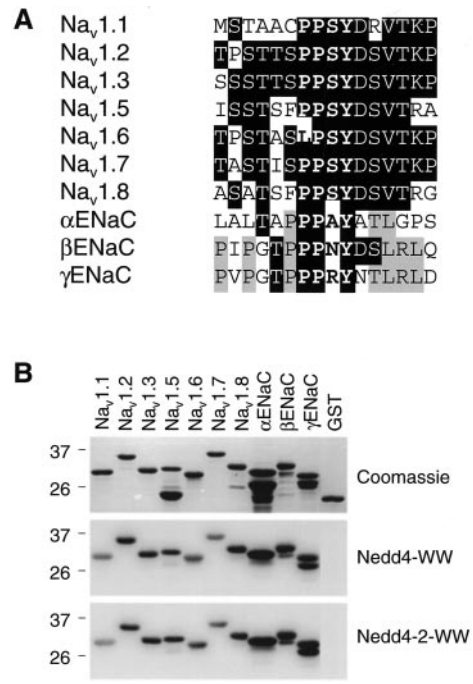

FIG. 1. **Na_v PY motif regions are highly homologous and interact with WW domains from Nedd4 and Nedd4-2.** *A*, alignment of core PY motif ((L/P)P*X*Y) and neighboring sequences (±6 amino acids) of mouse PY motif-containing Na_vs and the ENaC subunits. PY motifs are in *boldface*, and amino acids of the highest homology are in *white lettering* with *black background*. Amino acid positions of homology confined to ENaC subunits have a *gray background*. *B*, Far-Western analysis of binding between Na_vs and ENaC subunit PY motif-containing regions and WW domains of Nedd4 and Nedd4-2. Approximately 2 μg of each affinity-purified Na_v or ENaC carboxyl-terminal GST fusion protein was separated by SDS-PAGE. *The top panel* shows Coomassie Blue-stained gel of GST fusion proteins as indicated. Proteins were transferred to PVDF membranes and hybridized to ^{32}P-labeled Nedd4 or Nedd4-2 protein probes as indicated on the *right-hand side* of the gels. Molecular mass markers in kilodaltons are indicated on the *left-hand side* of the gels.

RESULTS

Several Na_v Channels Contain PY motifs in Their Carboxyl-terminal Regions—We analyzed amino acid sequences of mouse Na_v channel α-subunits to identify PY motif-containing channels. Conserved PY motifs conforming to the PP*X*Y consensus were identified in both human and mouse $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.5$, $Na_v1.7$, and $Na_v1.8$. $Na_v1.6$ was found to contain a PY motif variant (LP*X*Y) that has been shown to act as a ligand for Nedd4 WW domains (16) and represents a previously unidentified PY motif-containing Na_v channel. $Na_v1.4$ and $Na_v1.9$ do not contain PY motifs. An alignment of Na_v

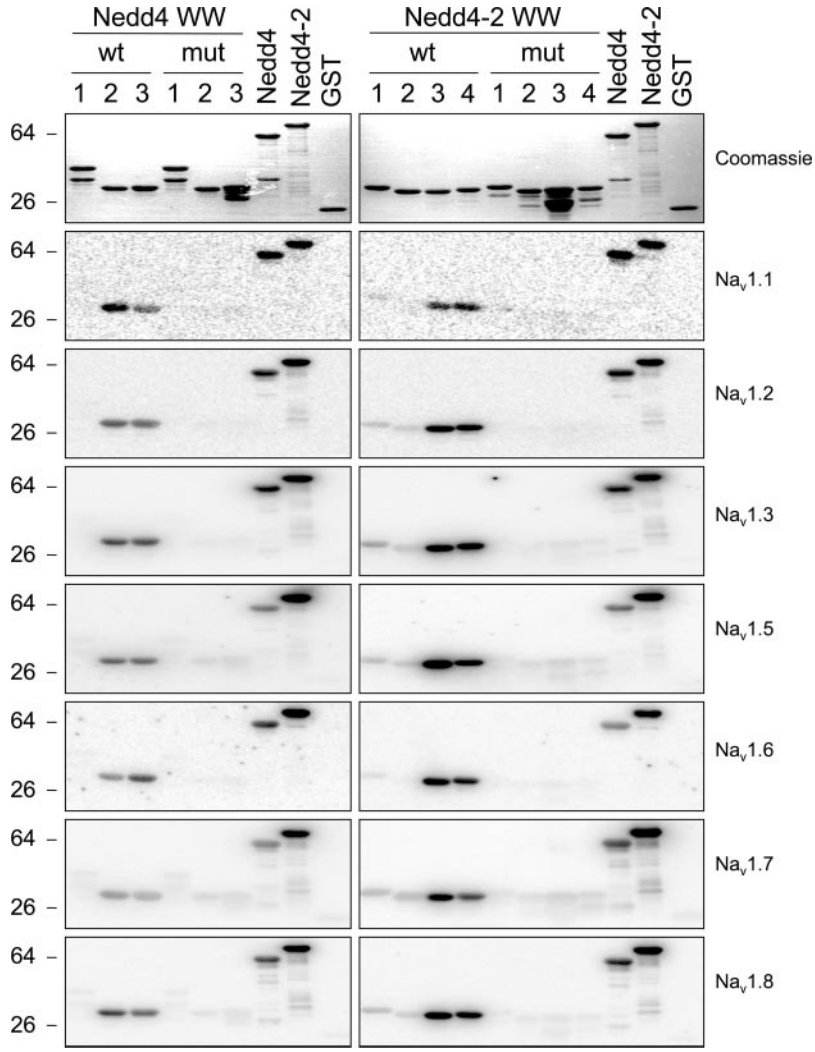

FIG. 2. **All PY motif-containing Na_v carboxyl termini interact with WW2 and WW3 of Nedd4 and WW3 and WW4 of Nedd4-2.** Far-Western analysis of binding between individual WW domains of Nedd4 and Nedd4-2 and Na_v carboxyl termini is shown. Approximately 2 μg of each affinity-purified GST-WW fusion protein was separated by SDS-PAGE. *Top*, Coomassie Blue-stained gel of GST fusion proteins as indicated above the gel; *wt* represents wild-type WW domains, and *mut* represents binding-deficient WW domain mutants. The *lanes* marked *Nedd4* and *Nedd4-2* represent WW1–3 of Nedd4 and WW1–4 of Nedd4-2, respectively. Proteins were transferred to PVDF membranes and hybridized to ^{32}P-labeled Na_v protein probes as indicated on *right-hand side* of the gels. Molecular mass markers in kilodaltons are indicated on the *left-hand side* of gels.

channel PY motif regions revealed a high degree of homology (Fig. 1*A*), suggesting that they may share similar WW domain binding specificity. Outside the PY motif itself, the homology between the PY motif-containing regions of the three ENaC subunits and the various Na_v α-subunits was limited (Fig. 1*A*).

Nedd4 and Nedd4-2 WW Domains Interact With the Carboxyl Termini of Seven Na_v Channels—To investigate whether interactions occur between PY motif-containing Na_v channels and Nedd4 or Nedd4-2 WW domains, a Far-Western approach was employed. The carboxyl termini of PY motif-containing Na_v channels were cloned from mouse cDNA and expressed as GST fusion proteins. Equal amounts of affinity-purified GST-Na_v proteins were electrophoresed on SDS-PAGE, immobilized on PVDF membranes, and then probed with ^{32}P-labeled Nedd4 or Nedd4-2 proteins containing their entire WW domain regions. Nedd4 and Nedd4-2 proteins interacted with all of the seven Na_v proteins tested to varying degrees but not with GST alone (Fig. 1*B*). Differences in affinity toward individual Na_v proteins were similar for both Nedd4 and Nedd4-2 probes; in each case the binding of Na_v1.1, Na_v1.6, or Na_v1.7 was 30–50% weaker than that of the other Na_v proteins (Fig. 1*B*). This was reproducible and was verified by semi-quantitative binding analysis.[2] As expected, both Nedd4 and Nedd4-2 WW domain-GST fusion proteins bound the PY motif containing ENaC subunits. It has been suggested that an extended PY motif containing a leucine residue three positions carboxyl to the tyrosine residue (PP*XYXXX*L) is a requirement for ENaC subunits to interact properly with Nedd4 WW domains (17). In the present work, we found that Nedd4 and Nedd4-2 WW domains interact with Na_v PY motifs that each have a valine residue in this position. This discovery, taken together with the finding that Na_v1.6, which has an LP*XY* motif (Fig. 1*A*), can bind Nedd4 and Nedd4-2 WW domains suggests that an extended consensus sequence of (L/P)P*XYXX*(L/V) in Na^+ channels is recognized by Nedd4 family WW domains.

The Na_v Channel PY Motifs Have Conserved WW Domain Binding Specificity—Previous studies have shown that interactions between ENaC and Nedd4 or Nedd4-2 are mediated by specific WW domains (10, 13). WW2 and WW3 of mouse Nedd4 (WW2, WW3, and WW4 of human Nedd4) and WW3 and WW4 of Nedd4-2 interact with each ENaC subunit in a PY motif-WW domain-specific manner *in vitro* (10, 13). To test which Nedd4 and Nedd4-2 WW domains mediate interactions with Na_v proteins, GST fusion proteins containing wild type and mutated individual WW domains of mouse Nedd4 and Nedd4-2 were generated. Equal amounts of the affinity-purified GST-WW domain proteins were immobilized on PVDF membranes after separation by SDS-PAGE and then probed with the ^{32}P-labeled carboxyl termini of PY motif-containing Na_v proteins. As shown in Fig. 2, proteins containing the entire WW domain region of Nedd4 and Nedd4-2 both interacted with each Na_v protein tested. However, binding was consistently stronger between the Na_v protein and Nedd4-2 than with Nedd4. Semi-quantitative binding analysis using ImageQuant software in-

[2] A. B. Fotia and S. Kumar, unpublished data.

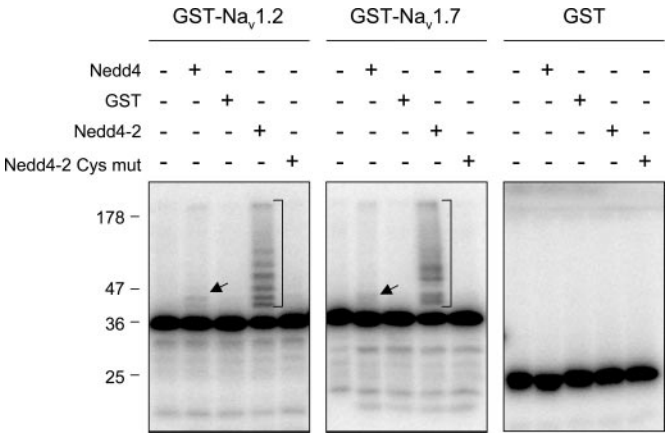

FIG. 3. **Nedd4-2 ubiquitinates GST-Na$_v$1.2 and GST-Na$_v$1.7 carboxyl termini *in vitro*.** ^{32}P-labeled Na$_v$1.2 and Na$_v$1.7 carboxyl-terminal GST fusion proteins were incubated in reactions containing E1, E2 (UbcH5b), ubiquitin, and ATP with or without Nedd4, GST, Nedd4-2, or the Nedd4-2 Cys mutant (*mut*), as indicated, followed by SDS-PAGE. In the presence of Nedd4-2, higher molecular weight bands (indicated) accompany Na$_v$1.2 and Na$_v$1.7 proteins representing ubiquitinated species. In the presence of Nedd4, at least one higher molecular weight band (*arrow*) is observed above the Na$_v$1.2 and Na$_v$1.7 proteins. No ubiquitination of the Na$_v$1.2 or Na$_v$1.7 channel occurs when GST or the Nedd4-2 Cys mutant are used in place of Nedd4 or Nedd4-2, nor does ubiquitination of GST alone occur under any condition.

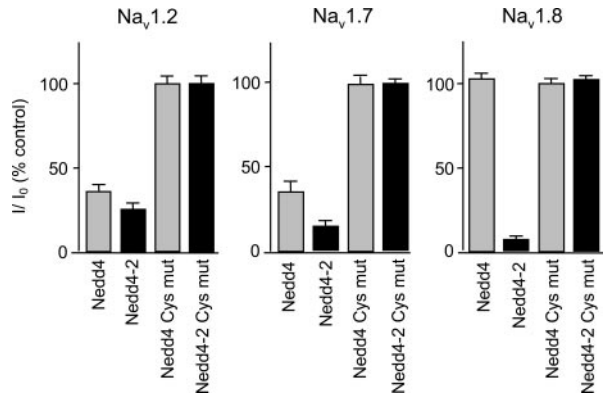

FIG. 4. **Effects of Nedd4, Nedd4-2, and Nedd4/Nedd4-2 Cys mutants on peak Na$^+$ current amplitude.** Bar graphs represent the percent changes in peak Na$^+$ current amplitude relative to the control (I/I_o) (oocytes expressing only the Na$_v$ channel α-subunit). Each Na$_v$ channel α-subunit was co-expressed in oocytes with either Nedd4, Nedd4-2, the Nedd4 Cys mutant (*mut*), or the Nedd4-2 Cys mutant. Data are expressed as the mean ± S.E. of 20–30 oocytes from 4–6 separate batches.

dicated that Nav1.5, Nav1.6, Nav1.7, and Nav1.8 binding to Nedd4-2 WW domains was at least 2-fold stronger than to Nedd4 WW domains, whereas binding differences were less pronounced for Nav1.1, Nav1.2, and Nav1.3 (data not shown).

Strikingly, all seven Na$_v$ proteins were repeatedly found to bind the same WW domains, namely WW2 and WW3 of mouse Nedd4 and WW3 and WW4 of Nedd4-2, but did not bind the inactive mutants of each of these domains (Fig. 2). This binding pattern is not restricted to the Na$_v$ proteins tested but is also seen with ENaC subunits (10, 13), suggesting a strong conservation of mechanism for Nedd4- and Nedd4-2-mediated regulation of sodium and perhaps other ion channels. Further analysis of the binding data showed that the Nedd4 WW2 domain binding was slightly stronger than the Nedd4 WW3 binding with all Na$_v$ channel α-subunits except Na$_v$1.6, which showed stronger binding to WW3. The Nedd4-2 WW3 and WW4 domains bound to essentially the same degree with Na$_v$1.1, Na$_v$1.2, and Na$_v$1.3; however, for Na$_v$1.5, Na$_v$1.6, Na$_v$1.7, and Na$_v$1.8 the Nedd4-2 WW3 domain showed 20–45% stronger binding than did the Nedd4-2 WW4 domain (data not shown).

*Nedd4-2 Ubiquitinates Na$_v$1.2 and Na$_v$1.7 Carboxyl Termini in Vitro*—The regulation of Na$_v$ channels by Nedd4 and Nedd4-2 potentially occurs by ubiquitination followed by degradation. PY motif-containing Na$_v$ channel α-subunits are generally lysine-rich at their intracellular carboxyl termini, indicating possible targets for ubiquitin conjugation. An *in vitro* ubiquitination assay was used to test this possibility. We incubated ^{32}P-labeled carboxyl-terminal GST fusions of Na$_v$1.2, a central nervous system-specific channel, and Na$_v$1.7, a peripheral nervous system-specific channel, with catalytically active recombinant Nedd4 or Nedd4-2 proteins in a ubiquitination buffer. Because of difficulties in expressing full-length Nedd4-2, a truncated form of protein containing the WW3, WW4, and HECT domains was used. This protein contains the ligand (Na$_v$ and ENaC) and E2 binding regions and the catalytic domain. In the presence of Nedd4-2, higher molecular mass bands representing ubiquitinated forms were observed for both Na$_v$ proteins (Fig. 3). However, in the presence of Nedd4 the ubiquitinated forms of Na$_v$1.2 and Na$_v$1.7 were barely detectable (Fig. 3). GST alone or a mutated Nedd4-2 protein lacking ubiquitin-protein ligase activity had no effect (Fig. 3). These data indicate that Nedd4-2-mediated regulation of Na$_v$s may occur via ubiquitination at the intracellular carboxyl-terminal region. Given that Nedd4 had only a weak ubiquitination effect on Na$_v$1.2 or Na$_v$1.7, Nedd4-mediated regulation of Na$_v$ channels may occur by either ubiquitination elsewhere within the Na$_v$ channel α-subunit or by some other mechanism. In separate assays with a known Nedd4 substrate, purified Nedd4 and Nedd4-2 proteins had equivalent ubiquitin-protein ligase activities.[2] Other lysine-rich regions exist in some Na$_v$ channels, which may serve as targets of ubiquitination by Nedd4. Because of the inherent difficulties in expressing full-length Na$_v$ channel α-subunits, attempts to produce larger Na$_v$ proteins containing additional lysine-rich regions for ubiquitination assays were unsuccessful.

Effects of Nedd4/4–2 on Na$_v$ Channel α-Subunit-mediated Na$^+$ Currents in Oocytes—*Xenopus* oocytes were used to investigate the effects of Nedd4 and Nedd4-2 on depolarization-activated Na$^+$ currents mediated by representative central nervous system and peripheral nervous system Na$_v$ channels. Control oocytes expressed only the cRNA for the corresponding Na$_v$ α-subunits. We observed that the expression of Nedd4 had differential effects on the Na$^+$ currents mediated by the various Na$_v$ channel α-subunits. Nedd4 had no effect on the peak Na$^+$ current amplitudes mediated by Na$_v$1.8 (105.4 ± 10.7%; $n = 20$) (Figs. 4 and 5). Increasing the amount of Nedd4 cRNA injected per oocyte to 50 ng also had no effect on Na$^+$ current amplitude (data not shown). However, when Nedd4 was co-expressed with Na$_v$1.7, we observed a significant reduction in the peak Na$^+$ current amplitudes to 35.8 ± 6.2% ($n = 20$; $p < 0.001$) of control current. Furthermore, Nedd4 had an equally pronounced effect on Na$_v$1.2, reducing the Na$^+$ current amplitude to 36.2 ± 3.9% ($n = 22$; $p < 0.001$) of control current (Figs. 4 and 5). A catalytically inactive Cys mutant of Nedd4 had no significant effect on Na$^+$ current amplitudes mediated by Na$_v$1.2 (100.9 ± 4.5%; $n = 20$), Na$_v$1.7 (100.4 ± 4.3%; $n = 20$), or Na$_v$1.8 (102.3 ± 9.9%; $n = 15$) (Fig. 4).

In contrast, Nedd4-2 effectively abolished the Na$^+$ currents mediated by these three Na$_v$ channel α-subunits (Figs. 4 and 5). The Na$_v$1.2-mediated Na$^+$ currents were reduced to 25.3 ± 3.7% ($n = 25$; $p < 0.001$), Na$_v$1.7 currents were reduced to 14.3 ± 3.1% ($n = 20$; $p < 0.001$), and Na$_v$1.8 currents were reduced to 6.8 ± 8.8% ($n = 30$; $p < 0.001$) of control currents. When oocytes were injected with 10-fold less Nedd4-2 cRNA, the inhibition of the Na$^+$ currents was not as pronounced,

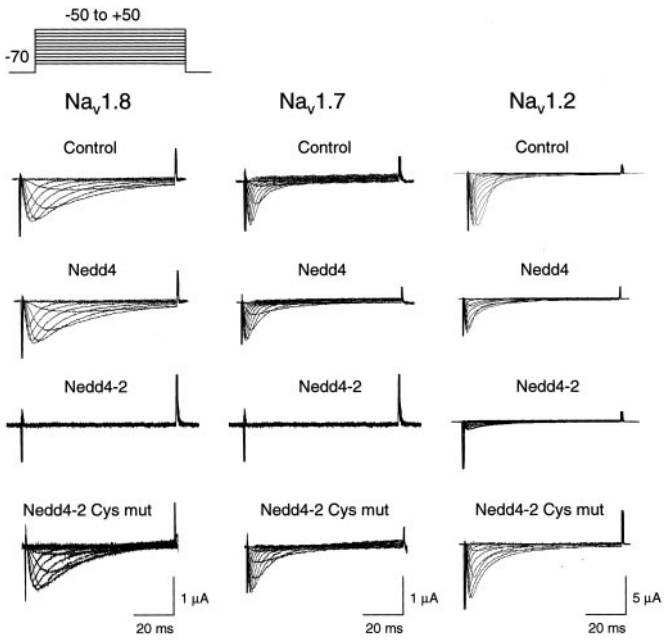

FIG. 5. **Effects of Nedd4, Nedd4-2, and the Nedd4-2 Cys mutant on Na_v channel α-subunit-mediated Na^+ currents in *Xenopus* oocytes.** Representative current traces showing the effects of Nedd4/4-2 and the Nedd4-2 Cys mutant (*mut*) on Na_v channel α-subunit-mediated Na^+ currents. Oocytes were held at −70 mV and depolarized to voltages of between −50 and +50 mV to evoke the inward Na^+ currents.

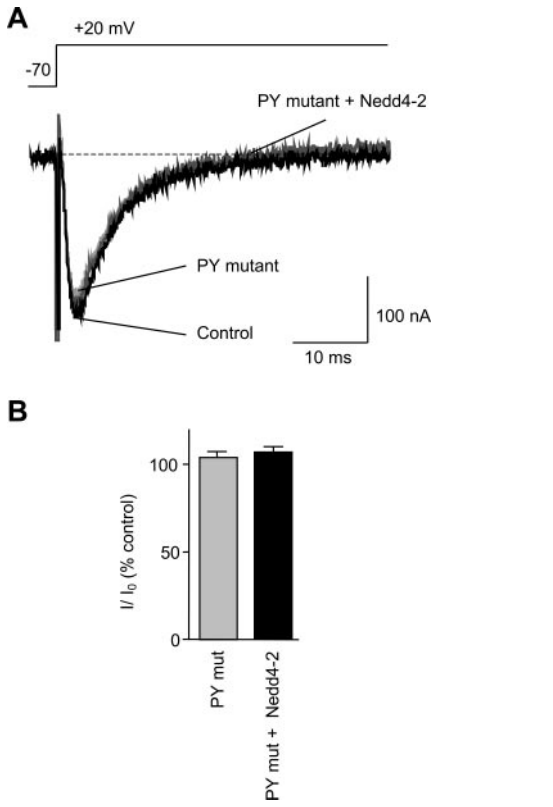

FIG. 6. **Effect of mutation of the PY motif on Na^+ currents mediated by Na_v1.8.** *A*, superimposed traces of depolarization-activated Na^+ currents obtained from *Xenopus* oocytes 2 days post-injection of mRNA for wild-type Na_v1.8 (*Control*), the PY mutant Na_v1.8, and the PY mutant in combination with Nedd4-2. Oocytes were held at −70 mV and depolarized to +20 mV to evoke an inward Na^+ current. *B*, bar graph representing the percent changes in peak Na^+ current amplitude relative to the control (I/I_o) (oocytes expressing wild type Na_v1.8 α-subunit). Oocytes were injected with mRNA for the PY mutant (*mut*) Na_v1.8 alone or in combination with Nedd4-2. Data are expressed as the mean ± S.E. of 30 oocytes from three separate batches.

demonstrating that the effect was directly proportional to the amount of Nedd4-2 expressed (data not shown). Finally, we confirmed that the inhibition of the Na^+ currents was due to Nedd4-2 ligase activity. Oocytes were injected with cRNA for the Cys mutant of Nedd4-2. In the presence of the ligase-deficient Nedd4-2 there was no effect on the peak Na^+ current amplitudes (n = 20–30) (Figs. 4 and 5).

To further test whether the functional interactions between Na_v channels and Nedd4/Nedd4-2 is mediated via the PY motifs in the carboxyl termini of these channels, we mutated this motif in Na_v1.8 (PPSY) by replacing Tyr-1921 with an Ala (Y1921A). As shown in Fig. 6, the mutated channel was no longer inhibited by Nedd4-2 in *Xenopus* oocytes, indicating that PY motif is required for Nedd4-2-mediated regulation of Na_v1.8 (n = 30).

DISCUSSION

Our data clearly demonstrate that all PY motif-containing Na_v channels can bind Nedd4 and Nedd4-2. We have further shown that all Na_v channels bind with the same WW domains (WW2 and WW3 of mouse Nedd4 and WW3 and WW4 of Nedd4-2) just as for ENaC subunits (10, 13). This is a striking similarity given that Na_v channels and ENaC constitute two different classes of Na^+ channels. We also found that subtle differences in WW domain preference occur between WW2 and WW3 of Nedd4 and WW3 and WW4 of Nedd4-2 for the tested Na_v channels. This may indicate a secondary role for the weaker interacting WW domain in each case, perhaps in binding to adaptor molecules.

We have also shown that the two Na_v channel α-subunits tested can be ubiquitinated by Nedd4-2 and that the activities of all three Na_v channels tested are inhibited by Nedd4-2. Using a PY motif mutant of Na_v1.8 we have further established that a functional interaction between the Na_v and the ubiquitin protein ligase is necessary for the inhibition of channel activity by the Nedd4-2 protein.

Interestingly, although all Na_v channels can bind WW domains from both Nedd4 and Nedd4-2, Nedd4 does not appear to ubiquitinate Na_v1.2 and Na_v1.7, the two Na_v channels tested in ubiquitination assays. On the other hand, in *Xenopus* oocytes the ectopic expression of Nedd4 can inhibit Na_v1.2 and Na_v1.7, although slightly less effectively than does Nedd4-2. These data suggest that Nedd4-2 and Nedd4 may differentially regulate various Na_v channels *in vivo*. It is possible that, whereas Nedd4-2 directly ubiquitinates and regulates Na_v channels, Nedd4 is involved in the ubiquitination of other proteins that regulate channel endocytosis and/or trafficking. Given that seven of the nine Na_v channels contain PY motifs in their carboxyl termini, we therefore propose that Na_v channels containing a PY motif are regulated by Nedd4/Nedd4-2-mediated ubiquitination.

Trafficking and recycling are known to play important roles in the short term regulation of many ion channels; however, little is known regarding the regulation of Na_v channel activity by membrane insertion and retrieval. The main evidence for short term down-regulation of Na_v channels comes from studies in fetal rat brains, where activation of the channels by agonists such as scorpion α-toxin or veratridine induces the internalization of the Na_v channels (18, 19). This channel down-regulation was mimicked simply by elevating intracellular Na^+, demonstrating that the agonists induced a negative feedback effect due to elevated intracellular Na^+ as a result of Na_v channel activation (18, 19). The mechanism underlying this effect remains unknown but appears to involve endocytotic/lysosomal compartments (20) that may be mediated by Nedd4/Nedd4-2-dependent ubiquitination of Na_v channels.

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Regulation of Neuronal Voltage-gated Sodium Channels by the Ubiquitin-Protein Ligases Nedd4 and Nedd4-2

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