Am J Physiol Cell Physiol 288: C692–C701, 2005.
First published November 17, 2004; doi:10.1152/ajpcell.00460.2004.

Molecular determinants of voltage-gated sodium channel regulation by the Nedd4/Nedd4-like proteins

Jean-Sébastien Rougier,[1,*] Miguel X. van Bemmelen,[1,*] M. Christine Bruce,[3,4] Thomas Jespersen,[1] Bruno Gavillet,[1] Florine Apothéloz,[1] Sophie Cordonier,[1] Olivier Staub,[1] Daniela Rotin,[3,4] and Hugues Abriel[1,2]

[1]*Department of Pharmacology and Toxicology, University of Lausanne;* [2]*Service of Cardiology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland;* [3]*The Hospital for Sick Children, Toronto; and* [4]*Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada*

Submitted 20 September 2004; accepted in final form 11 November 2004

Rougier, Jean-Sébastien, Miguel X. van Bemmelen, M. Christine Bruce, Thomas Jespersen, Bruno Gavillet, Florine Apothéloz, Sophie Cordonier, Olivier Staub, Daniela Rotin, and Hugues Abriel. Molecular determinants of voltage-gated sodium channel regulation by the Nedd4/Nedd4-like proteins. *Am J Physiol Cell Physiol* 288: C692–C701, 2005. First published November 17, 2004; doi:10.1152/ajpcell.00460.2004.—The voltage-gated Na^+ channels (Na_v) form a family composed of 10 genes. The COOH termini of Na_v contain a cluster of amino acids that are nearly identical among 7 of the 10 members. This COOH-terminal sequence, PPSYDSV, is a PY motif known to bind to WW domains of E3 protein-ubiquitin ligases of the Nedd4 family. We recently reported that cardiac $Na_v1.5$ is regulated by Nedd4-2. In this study, we further investigated the molecular determinants of regulation of Na_v proteins. When expressed in HEK-293 cells and studied using whole cell voltage clamping, the neuronal $Na_v1.2$ and $Na_v1.3$ were also downregulated by Nedd4-2. Pull-down experiments using fusion proteins bearing the PY motif of $Na_v1.2$, $Na_v1.3$, and $Na_v1.5$ indicated that mouse brain Nedd4-2 binds to the Na_v PY motif. Using intrinsic tryptophan fluorescence imaging of WW domains, we found that $Na_v1.5$ PY motif binds preferentially to the fourth WW domain of Nedd4-2 with a K_d of ~55 μM. We tested the binding properties and the ability to ubiquitinate and downregulate $Na_v1.5$ of three Nedd4-like E3s: Nedd4-1, Nedd4-2, and WWP2. Despite the fact that along with Nedd4-2, Nedd4-1 and WWP2 bind to $Na_v1.5$ PY motif, only Nedd4-2 robustly ubiquitinated and downregulated $Na_v1.5$. Interestingly, coexpression of WWP2 competed with the effect of Nedd4-2. Finally, using brefeldin A, we found that Nedd4-2 accelerated internalization of $Na_v1.5$ stably expressed in HEK-293 cells. This study shows that Nedd4-dependent ubiquitination of Na_v channels may represent a general mechanism regulating the excitability of neurons and myocytes via modulation of channel density at the plasma membrane.

ubiquitin; Nedd4-2; PY motif; $Na_v1.5$; human *ether-à-go-go*-related gene

VOLTAGE-GATED SODIUM CHANNELS (Na_v) are membrane proteins critical for the initiation and propagation of action potentials in excitable cells such as neurons and cardiac and skeletal myocytes (4). These channels consist of one α-subunit with an apparent molecular mass of ~260 kDa associated with small ancillary β-subunits of ~35 kDa (12). The α-subunit is the pore-forming protein and is sufficient for functional expression in heterologous expression systems. The human genome contains 10 genes encoding α-subunits ($Na_v1.1$–$Na_v1.9$ and Na_x), which are expressed mostly in excitable cells (6). Interestingly, cells such as neurons and cardiomyocytes may simultaneously express several members of the Na_v family, and in most cases, the specific role played by these different isoforms is not clear. Importantly, abnormal function of Na_vs due to naturally occurring mutations in genes coding for $Na_v1.1$, $Na_v1.2$, $Na_v1.4$, and $Na_v1.5$ cause severe neurological and cardiac disorders in humans (22).

Thus far, little is known about the molecular determinants of trafficking, targeting, sorting, and internalization of Na_v. Recently, two studies provided molecular evidence that Na_v may be targets of ubiquitin-protein ligases of the Nedd4/Nedd4-like family (5, 31). At least nine genes coding for such Nedd4/Nedd4-like enzymes have been found in the human genome (11). Several of the members of this family are found in the nervous system, where they have been proposed to play a role in central nervous system development and axon guidance (20, 25). These proteins are ubiquitin-protein ligases (E3) comprising a C2 domain, two to four WW domains, and a catalytic Hect domain (7, 27). The C2 domain is involved in membrane targeting, the WW domains are protein-protein interaction modules involved in substrate recognition, and the Hect domain is responsible for catalytic ubiquitin ligase (E3) activity. Thus far, besides Na_v, two other ion channels mainly expressed in epithelial cells, i.e., the epithelial Na^+ channel (ENaC) and the Cl^- channel ClC5, have been shown to be regulated by Nedd4-2 (2) and WWP2 (28), respectively. Both E3s belong to the family of Nedd4/Nedd4-like proteins (11). The current working model proposes that upon binding of Nedd4/Nedd4-like proteins to their target membrane ion channels, the latter are ubiquitinated and thereafter internalized and/or degraded. The interaction between the Nedd4/Nedd4-like proteins and their targets is mediated by WW domains of the E3s and a PY motif that, with the exception of $Na_v1.4$ and $Na_v1.9$, is found in the COOH termini of Na_vs and ENaCs (Fig. 1). The canonical PY motif is represented by the minimal sequence (L/P)PxY (17). In the case of ClC5, the sequence shown to be important for Nedd4/Nedd4-like protein regulation is very similar, i.e., PPLPPY (28). In addition, in recent reports (9, 16), researchers have presented evidence that a hydrophobic residue in position +3 after the Tyr (Tyr+3) of the PY motif is involved in the binding to the WW-domain pocket, hence forming an "extended" PY motif with the sequence (L/P)PxYxxϕ, with ϕ being a hydrophobic residue. Interestingly,

* J.-S. Rougier and M. X. van Bemmelen contributed equally to this work.
Address for reprint requests and other correspondence: H. Abriel, Dept. of Pharmacology and Toxicology, Service of Cardiology, Univ. of Lausanne, Rue du Bugnon 27, CH-1005 Lausanne, Switzerland (E-mail: Hugues.Abriel@unil.ch).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

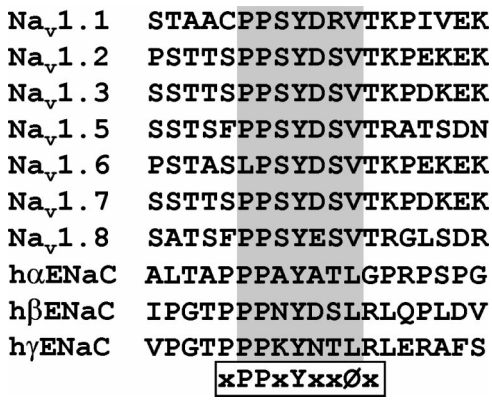


Fig. 1. Alignment of the PY motif of voltage-gated Na^+ channels (Na_v) and epithelial Na^+ channel (ENaC) subunits. The extended PY motifs (shaded sequences) are found in the cardiac Na_v ($Na_v1.5$) and most neuronal Na_v isoforms. They are absent in $Na_v1.4$ and $Na_v1.9$. Note that in the position +3 downstream from the central Tyr, Na_vs have a Val and ENaC subunits have a Leu, both of which are hydrophobic residues, consistent with the proposed sequence of an extended PY motif (boxed sequence).

such a sequence is present and conserved among 7 of the 10 Na_v α-subunits as well as in all three ENaC subunits (Fig. 1).

In this study, we further investigated the molecular determinants of the regulation of Na_v isoforms by Nedd4/Nedd4-like ubiquitin ligases. We mainly used the cardiac $Na_v1.5$ isoform as a prototype for the other members of the Na_v family. We have shown that, besides $Na_v1.5$, $Na_v1.2$ and $Na_v1.3$ also are negatively regulated by Nedd4-2 when coexpressed in mammalian cells. In this report, we provide evidence that not only Nedd4-2 but also Nedd4-1 and WWP2 bind to the PY motif of $Na_v1.5$. However, ubiquitination and downregulation of $Na_v1.5$ was much weaker with Nedd4-1 and WWP2. Finally, we present data suggesting that WWP2 may compete with the Nedd4-2 effects when both are coexpressed and that Nedd4-2 decreases the $Na_v1.5$ density at the cell membrane by increasing the internalization rate.

MATERIALS AND METHODS

DNA constructs and cell lines. Human $Na_v1.5$ and rat $Na_v1.2$ cDNA were gifts from Dr. R. S. Kass (Columbia University, New York, NY) and Dr. T. Scheuer (University of Washington, Seattle, WA), respectively. Human Nedd4-1 (KIAA0093) and Nedd4-2 (KIAA0439) cDNA were gifts from Dr. T. Nagase (Kazusa Institute, Japan). Human WWP2 cDNA (GenBank accession no. U96114) was amplified using PCR from a human heart cDNA library (Matchmaker, NT 91-2769; Clontech, Basel, Switzerland), cloned into *Eco*RI-digested pCDNA3.1 (Invitrogen), and contained a T7 epitope in the NH_2 terminus inserted using PCR. All constructs were cloned into pcDNA3.1 vector (Invitrogen). All Na_v and Nedd4 mutant constructs were generated using the QuickChange mutagenesis kit (Stratagene, Amsterdam, The Netherlands) and verified by sequencing. The human embryonic kidney (HEK)-293 cell line stably expressing human $Na_v1.3$ was a kind gift from GlaxoSmithKline (Brentford, UK), and the HEK-293 cell line stably expressing human $Na_v1.5$ was previously described (23).

Antibodies. Rabbit serum against human $Na_v1.5$ COOH terminus, raised against a glutathione *S*-transferase (GST) fusion protein comprising residues 1,978–2,016, was a gift from Alomone Laboratories (Jerusalem, Israel) and was characterized previously (31). SP19 anti-pan-Na_v rabbit polyclonal antibody was obtained from Upstate (Waltham, MA), anti-ubiquitin monoclonal FK2 antibody was from Affiniti Research (Exeter, UK), and anti-Nedd4-1 and anti-Nedd4-2 antibodies were described previously (14, 30). Anti-T7 and anti-actin antibodies were obtained from Sigma (Buchs, Switzerland).

Transfection and homogenization of HEK-293 cells. HEK-293 cells, either nontransfected or stably expressing $Na_v1.5$, were transiently transfected with Nedd4-2 constructs using Ca^{2+}-phosphate. Two days after transfection, cells were solubilized by 30-min rotation at 4°C in lysis buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, and Complete protease inhibitor cocktail (1 tablet/25 ml; Roche, Rotkreuz, Switzerland). Soluble fractions were recovered in supernatants after 15 min of centrifugation at 18,000 *g*. Protein content was measured using Bradford test-based CooAssay reagent (Uptima, Basel, Switzerland) with BSA as a reference.

Brain tissue preparation. Brains of 3- to 4-month-old mice (C57BL/6 strain, bred in-house) were excised and transferred to lysis buffer containing (in mM) 20 HEPES, pH 7.6, 125 NaCl, 10% glycerol, 1 EGTA, 1 EDTA, 1 dithiothreitol (DTT), 1 PMSF, and Complete protease inhibitor cocktail (Roche). Tissue was homogenized using a Polytron and a Teflon homogenizer. Triton X-100 was added to a final concentration of 1%, and solubilization was induced by rotating for 1 h at 4°C. The soluble fraction from 15-min centrifugation at 13,000 *g* (4°C) was used as a source of Nedd4-2 for pull-down assays. Animal experiments were performed in accordance with Swiss law.

Pull-down assays. The cDNA encoding the last 57, 56, and 66 amino acids of rat $Na_v1.2$, human $Na_v1.3$, and $Na_v1.5$, respectively, were cloned into pGEX-4T1 (Amersham, Otelfingen, Switzerland). In addition, we generated Tyr-to-Ala PY-motif mutants of each construct. Expression of GST fusion proteins in *Escherichia coli* cells was induced with 0.2 mM isopropylthiogalactoside for 3 h at 30°C. Cells were harvested by centrifugation and resuspended in lysis buffer. Soluble fractions from a 15-min centrifugation at 13,000 *g* (4°C) were rotated for 1 h in the presence of glutathione (GSH)-Sepharose at 4°C. Beads containing bound fusion proteins were recovered after washing and used in pull-down experiments. Amounts (2 μg) of GST fusion proteins used in the pull-downs were verified by fluorescent staining of proteins in gels using the Insite dye (National Diagnostics, Basel, Switzerland). GST pull-down assays of soluble fractions of brain lysate were performed using GSH-Sepharose beads containing either GST or one of the GST fusion proteins. After overnight incubation and washing of the beads (in mM: 20 HEPES, pH 7.6, 500 NaCl, 1% Triton X-100, and 1 PMSF), bound Nedd4-2 was detected using Western blot analysis.

Detection of ubiquitinated $Na_v1.5$. To study the ubiquitination of $Na_v1.5$ by Nedd4-like proteins, HEK-293 cells were transfected with either empty vector or wild-type (WT) or mutant Na_v alone or together with Nedd4-2, Nedd4-1, or T7-tagged WWP2. Cells were solubilized as described previously in lysis buffer supplemented with 10 mM *N*-ethylmaleimide. Samples (1 ml) of soluble fractions containing 1 mg of protein were incubated for 2 h under rotation at 4°C in the presence of 2 μl of anti-$Na_v1.5$ serum. After the addition of 25 μl of protein A-Sepharose (drained volume), the protein solutions were incubated for an additional 1 h as before. After extensive washing, bound proteins were eluted by 5 min of boiling of the beads in 50 μl of sample buffer containing 50 mM DTT, and ubiquitination levels were determined by performing Western blot analysis using FK2 antibody.

K_d measurement. Peptides representing sequences of human $Na_v1.5$ and human *ether-à-go-go*-related gene (hERG) were synthesized by the Hospital for Sick Children/Advances Protein Technology Centre (Toronto, ON, Canada). The mass and purity of the peptides were confirmed by performing electrospray mass spectrometry. Peptide sequences were human $Na_v1.5$, STSFPPSYDSVTR, and hERG, QRMTLVPPAYSAVTT. Lyophilized peptides were resuspended in 150 mM KCl and 10 mM K^+-phosphate, pH 6.5. Peptide concentrations were measured in 6.0 M guanidine HCl at Ala^{280} (26). The WW domains of *Xenopus* Nedd4-2 (GenBank accession no. CAA03915), WW1 (residues 186–225), WW2 (residues 377–416), WW3 (residues

489–528), and WW4 (residues 540–579) were subcloned into pQE-30 and expressed as NH_2-terminal MRGS (methionine, arginine, glycine, serine)-His^6-tagged proteins. These WW domains are identical to the human Nedd4-2 WW domains. Proteins were expressed and purified from *E. coli* M15 pREP4 as described previously (9). Intrinsic tryptophan fluorescence of the WW domains was used to monitor peptide binding. Fluorescence measurements were obtained using a Hitachi F-2500 fluorescence spectrophotometer at 25°C with excitation and emission wavelengths of 298 and 333 nm, respectively, and slit width of 2.5 nm. Experiments were measured in 150 mM KCl and 10 mM K^+-phosphate, pH 6.5, with WW-domain concentrations kept constant at 2 μM. Peptides were added at concentrations ranging from 0 to 1.2 mM. Calculations of the equilibrium dissociation constant (K_d) were performed as described previously (9, 16).

Electrophysiology. For electrophysiological studies, HEK-293 cells were transiently cotransfected with 0.3 μg of $Na_v1.2$, $Na_v1.5$-WT, or mutant constructs and 1.4 μg of Nedd4-2, Nedd4-1, WWP2-WT, or mutant constructs or empty vector (control). HEK-293 cells stably expressing $Na_v1.3$-WT were transiently transfected with either Nedd4-2-WT or CS-mutated Nedd4-2 (i.e., Nedd4-2 in which Cys^{801} was mutated into a Ser) cDNA (1.4 μg) or empty vector. Na_v β-subunits were not cotransfected. All transfections included 0.8 μg of cDNA encoding CD8 antigen as a reporter gene. Cells were incubated for 18 h with the transfection Ca^{2+}-phosphate mix. After 24 h, cells were split at low density. Anti-CD8 beads (Dynal, Oslo, Norway) were used to identify transfected cells, and only decorated cells were analyzed.

Whole cell currents were measured at room temperature (22–23°C). The internal pipette solution was composed of (in mM) 60 CsCl, 70 Cs-aspartate, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 11 EGTA, and 5 Na_2-ATP, pH 7.2, with CsOH. The external solution contained (in mM) 130 NaCl, 5 CsCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, and 5 glucose, pH 7.4, with CsOH. Measurements were made using pClamp software, version 8 (Axon Instruments, Union City, CA) and a VE-2 amplifier (Alembic Instruments, Montreal, QC, Canada). Data were analyzed using pClamp software, version 8 (Axon Instruments), and KaleidaGraph software (Synergy Software, Reading, PA). Peak currents were measured using a current-voltage protocol, and Na^+ current (I_{Na}) densities (expressed as pA/pF) were obtained by dividing the peak current by the cell capacitance obtained using the pClamp function.

Confocal imaging. HEK-293 cells were transiently transfected with 0.025 μg of a $Na_v1.5$-yellow fluorescent protein (YFP) construct (33) (kind gift from Dr. T. Zimmer, University of Jena, Jena, Germany) and with Nedd4-2-WT or Nedd4-2-CS mutant (1.4 μg). Alternatively, to ascertain that the analyzed cells were transfected by both constructs, Nedd4-2-WT and -CS constructs that were tagged with green fluorescent protein (GFP) at the NH_2 terminus by subcloning Nedd4-2 into peGFP-C1 (Clontech) were also transfected. We observed no difference between the Nedd4-2 GFP-tagged and nontagged constructs. For these experiments, we had to reduce 10-fold the amount of transfected $Na_v1.5$ DNA compared with standard transfections, because under the latter conditions, the localization of the protein was restricted mainly to intracellular compartments (33). Two days after transfection, fluorescent proteins were visualized using confocal microscopy (LSM 510; Zeiss, Göttingen, Germany) with living cells. Optical sections were obtained at 512 × 1024-pixel resolution, and fluorescence intensities were analyzed using LSM software (Zeiss).

Statistical analysis. Data are represented as means ± SE. A two-tailed Student's *t*-test was used to compare the means.

RESULTS

$Na_v1.5$, $Na_v1.2$, and $Na_v1.3$ currents are downregulated by Nedd4-2. Investigators at our laboratory (31) recently provided evidence that the cardiac $Na_v1.5$ is ubiquitinated in cardiac tissues as well as when heterologously expressed in HEK-293 cells. Moreover, transfected Nedd4-2 was previously shown to downregulate I_{Na} in a HEK-293 cell line stably expressing $Na_v1.5$ (31). This effect was dependent on the integrity of the $Na_v1.5$ PY motif because channels in which Tyr^{1977} was changed to alanine were not regulated by Nedd4-2.

In the present study, we extended our investigations to two other Na_v members expressed in the nervous system that have a PY motif identical to that of $Na_v1.5$ (Fig. 1). For this purpose, we generated GST fusion proteins of the COOH termini of rat $Na_v1.2$, human $Na_v1.3$, and $Na_v1.5$, comprising their PY motifs, as well as mutant forms in which the Tyr of the PY motif was mutated into Ala. We performed pull-down experiments on mouse brain lysates using these fusion proteins, and as presented in Fig. 2*A*, at least two variants of Nedd4-2 expressed in mouse brain were pulled down in a PY-motif-dependent manner. Nedd4-2 was recovered to a similar extent with all three fusion proteins. These results are similar to those obtained with $Na_v1.5$ fusion proteins used in pull-down experiments using cardiac lysates (31). The presence of different sizes of Nedd4-2 in mouse brain may represent splice variants as recently reported for other tissues (13).

The effect of coexpressing Nedd4-2 on $Na_v1.2$ (Fig. 2, *B* and *C*) and $Na_v1.3$ (Fig. 2, *D* and *E*) currents was assessed by performing whole cell patch-clamp experiments using HEK-293 cells transiently transfected with rat $Na_v1.2$ and cells stably expressing human $Na_v1.3$. The results were similar for both channels (Fig. 2, *B–E*). Nedd4-2 robustly decreased the I_{Na} density by ~80–90%. This downregulation required the ubiquitin ligase activity of the enzyme because catalytically inactive Nedd4-2 (Nedd4-2-CS) did not reduce $Na_v1.2$ and $Na_v1.3$ I_{Na}. In the case of $Na_v1.3$, Nedd4-2-CS increased I_{Na} threefold, suggesting that the cell surface density of this channel isoform was regulated by an endogenous Nedd4-like activity. Similar results were obtained when studying the effect of Nedd4-2-CS on $Na_v1.5$ and ENaC expressed in *Xenopus* oocytes (1, 2), suggesting that the catalytically inactive Nedd4-2 may, in particular conditions, exert an antagonistic effect on endogenous Nedd4-like proteins.

Analysis of the extended PY motif of $Na_v1.5$ and its interaction with Nedd4-like proteins. In recent studies (9, 16), the affinities of the PY motifs of the three ENaC subunits for Nedd4 WW domains were determined by measuring the change in intrinsic tryptophan fluorescence of the WW domains upon binding of peptides. To determine the binding affinity of the PY motif of $Na_v1.5$ to WW domains of Nedd4, we performed similar experiments using a peptide comprising the PY motif of human $Na_v1.5$ (1970STSFPPSYDSVTR1982). To asses whether the presence of a consensus PY motif is sufficient for binding of a peptide to a WW domain, we also tested another PY-motif-containing peptide found in the COOH-terminal region of the cardiac delayed rectifier hERG channel (voltage-gated K^+ channel 11.1). The sequence of this peptide is 1069QRMTLVPPAYSAVTT1083.

The $Na_v1.5$ PY-motif peptide was able to bind to the fourth WW domain (WW4) of Nedd4-2 with moderate affinity (~55 μM), while its binding to each of the other three WW domains of Nedd4-2 was either very poor or undetectable (Table 1). This includes WW3, which was previously demonstrated to strongly bind the PY motif of β-ENaC (Ref. 9 and Table 1). WW4 of rat Nedd4-1 bound the $Na_v1.5$ peptide with somewhat lower affinity than the Nedd4-2 WW4 domain (Table 1), even though they share a high degree of sequence similarity. In

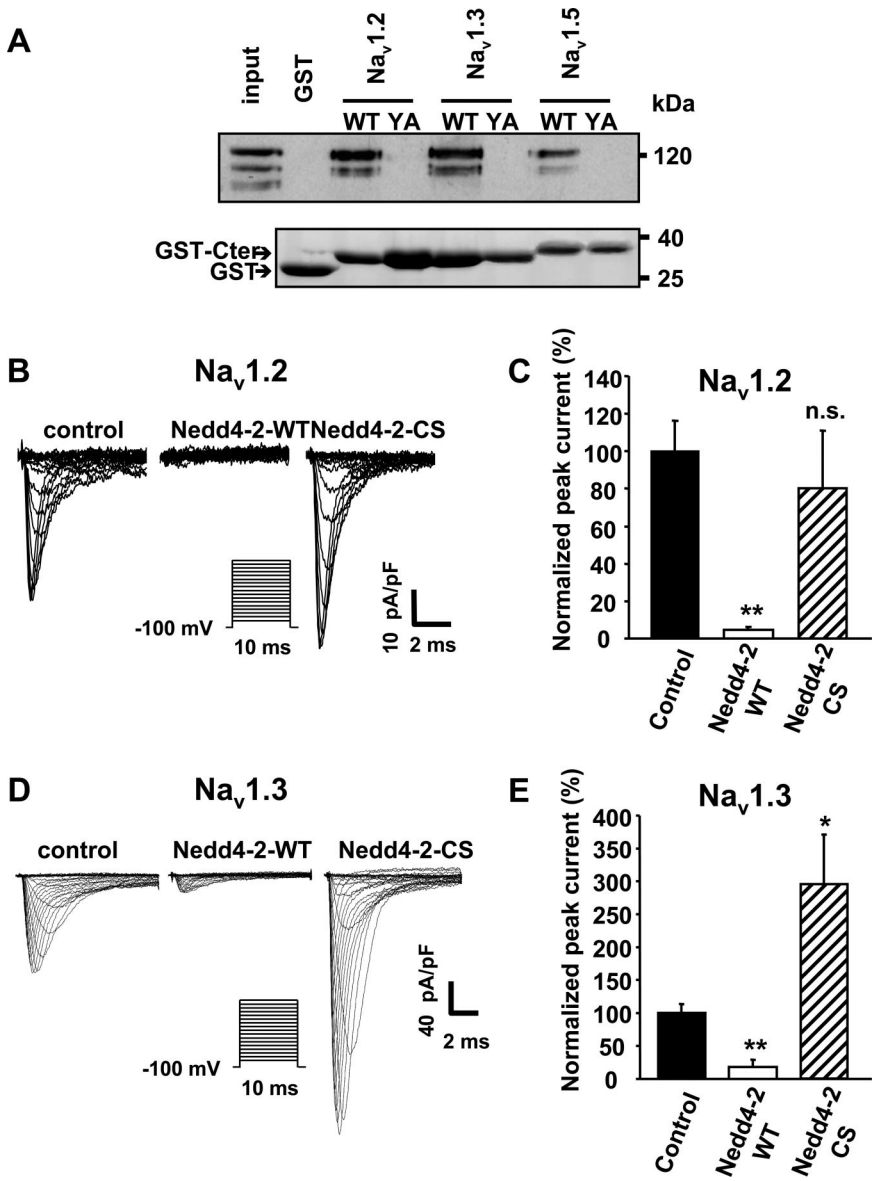


Fig. 2. Binding of brain Nedd4-2 to the PY motifs of $Na_v1.2$, $Na_v1.3$, and $Na_v1.5$, and Nedd4-2-dependent regulation of neuronal Na^+ current (I_{Na}) expressed in human embryonic kidney (HEK)-293 cells. *A*: mouse brain tissue was lysed as described in MATERIALS AND METHODS. Samples of soluble fractions were diluted in lysis buffer and mixed with GSH-Sepharose beads containing either glutathione *S*-transferase (GST) or each of the two GST COOH-termini fusion proteins (GST-Cter) [wild type (WT) or YA mutant] (*bottom*). Bound Nedd4-2 was detected using Western blot analysis. In the *input lane*, three bands were recognized by the specific Nedd4-2 antibody, and two of them were found to bind to the Na_v fusion proteins. *B* and *D*: whole cell recordings of HEK-293 cells expressing rat $Na_v1.2$ (*B*) and human $Na_v1.3$ (*D*) that were transiently transfected with either WT or the inactive CS-mutant Nedd4-2 (Nedd4-2 in which Cys^{801} was mutated into a Ser). *C* and *E*: bar graphs summarizing the Nedd4-2-dependent decrease in $Na_v1.2$ (*C*) and $Na_v1.3$ (*E*) peak I_{Na}. n = 8–9 cells from 2 experiments. $*P < 0.05$. $**P < 0.001$. n.s., nonsignificant.

contrast to the $Na_v1.5$ PY peptide, the PY peptide derived from the hERG channel bound very poorly to the WW domains of Nedd4-2 (Table 1), suggesting that subtle differences in the peptide sequence, besides the PPxYxxV consensus residues, can play an important role in these interactions. Moreover, the latter results are consistent with pull-down experiments that showed that GST-fusion proteins comprising the PY motif of hERG did not bind Nedd4-2 (Gavillet B and Abriel H, unpublished data).

Table 1. *Affinity of interactions between Nedd4 WW domains and the PY motifs of $Na_v1.5$, hERG, and β-ENaC*

	$Na_v1.5$-PY, μM	hERG-PY, μM	ENaC-βPY, μM
Nedd4-2, WW1	>400 (n=1)	>500 (n=1)	
Nedd4-2, WW2	NB (n=1)	>330 (n=1)	
Nedd4-2, WW3	NB (n=5)	>300 (n=4)	14.4±4 (n=3)
Nedd4-2, WW4	55.1±9.0 (n=6)	>900 (n=3)	
Nedd4-1, WW4	64.1±12.2 (n=2)		

Where indicated values are average ± SE, except *bottom row,* which represents an average error of 2 measurements. PY-motif peptides used: $Na_v1.5$, STSFPPSYDSVTR; hERG, QRMTLVPPAYSAVTT. Na_v, voltage-gated Na^+ channel; hERG, human *ether-à-go-go*-related gene; β-ENaC, β-subunit of epithelial Na^+ channel; NB, no binding.

Investigators at our laboratory (31) recently showed that, similarly to the PY motifs of ENaC subunits, the hydrophobic residue found in position +3 to the Tyr of the PY motif of $Na_v1.5$ plays a role in the interaction with Nedd4-2. These results suggest that the sequence of the PY motif that is important in the interaction with the WW domains of Nedd4-2 may be extended to the Tyr+3 position as previously proposed for β-ENaC PY motif (9, 16). To further investigate this possibility, we performed an alanine scan of the region encompassing the putative extended PY motif of $Na_v1.5$ by replacing all single residues between Pro^{1974} and Val^{1980} with Ala. With the use of structural analysis, the homologous region of ENaC was previously shown to be involved in binding to the WW domains (16). These point mutations were generated both in the DNA constructs used for the production of GST COOH-

terminal fusion proteins and for the expression of full-length $Na_v1.5$ proteins in HEK-293 cells. Figure 3*A* illustrates the results of the pull-down experiments performed with these mutant PY-motif fusion proteins using lysates of HEK-293 cells transiently transfected with Nedd4-2, Nedd4-1, or WWP2. These three proteins are members of the family of Nedd4-like protein-ubiquitin ligases (27) and have been shown to be expressed in cardiac tissues as well as in the nervous system. The results of these pull-down experiments indicate that the sequence requirements for binding of all three Nedd4-like proteins are very similar (Fig. 3*A*) as illustrated in the quantification of three such experiments (Fig. 3*B*). Replacement of $Na_v1.5$ Pro^{1974}, Pro^{1975}, and Tyr^{1977} with Ala strongly reduced the amount of bound Nedd4-like proteins. Interestingly, and in accordance with previous results obtained in our laboratory (31), replacement of the Val^{1980} with an Ala (also a hydrophobic residue) did not reduce the binding of any of the three ubiquitin ligases. Indeed, investigators at our laboratory (31) previously found that only the substitution of Val^{1980} with charged residues altered the binding of Nedd4-2 to the $Na_v1.5$ COOH terminus under pull-down conditions. An estimation of the relative binding affinity of the three E3s for the WT PY motif of $Na_v1.5$ can be obtained by comparing the amount of bound E3 with the quantity of the same E3 present in the cell lysate (Fig. 3*A*). Consistent with the K_d values that we obtained experimentally (Table 1), this ratio is in a range similar to that found for Nedd4-2 and Nedd4-1 as well as WWP2.

We next transiently expressed the corresponding seven mutant $Na_v1.5$ channels in HEK-293 cells to test whether these mutations might interfere with the Nedd4-2-dependent downregulation of $Na_v1.5$. All seven channels yielded I_{Na} that were not different from $Na_v1.5$-WT as assessed by whole cell patch clamping (data not shown). As presented in Fig. 3*C*, WT I_{Na} was robustly decreased upon coexpression of Nedd4-2, but this was not the case for the channels bearing the Ala mutations shown to abrogate the binding to Nedd4-2, i.e., Pro^{1974}, Pro^{1975}, and Tyr^{1977}. The Asp^{1978}, Ser^{1979}, and Val^{1980} to Ala mutations also impaired, albeit to a lower degree, Nedd4-2-mediated downregulation of $Na_v1.5$ I_{Na}. Only in the case of the Ser^{1976} mutation was Nedd4-2-dependent downregulation of I_{Na} comparable to that observed with WT channels.

Role of other ubiquitin-protein ligases of the Nedd4/Nedd4-like family. As presented above, we found that besides Nedd4-2, Nedd4-1 and WWP2 also bind to the COOH-termi-

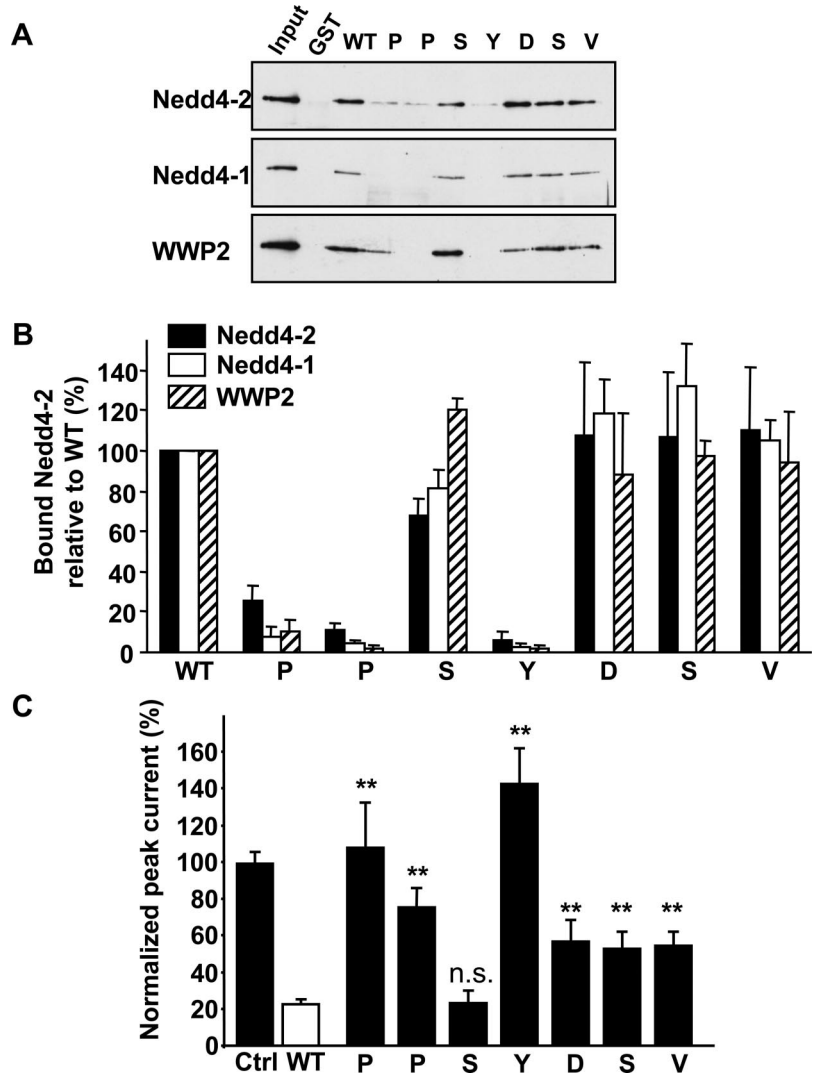


Fig. 3. Alanine scan of extended PY motif of $Na_v1.5$. *A*: GST fusion proteins of the last 66 residues of $Na_v1.5$ were generated as described in MATERIALS AND METHODS. WT and seven different mutant proteins in which all single residues comprising the extended PY motif were mutated into Ala and used in pull-down experiments with lysates of HEK-293 cells transiently expressing Nedd4-2, Nedd4-1, and T7-epitope-tagged WWP2. The amount (2 μg) of GST fusion proteins used in the pull-down experiments was verified by fluorescent staining of proteins in gels using the Insite dye (data not shown). The pull-down proteins were evaluated using Western blot analysis with Nedd4-2, Nedd4-1, and T7-epitope-specific antibodies. *B*: bar graph quantifying the amount of bound Nedd4-2, Nedd4-1, and WWP2 to the WT and alanine-mutant $Na_v1.5$ fusion proteins obtained in the three pull-down experiments. Values are normalized to levels of E3 bound to WT fusion proteins. *C*: effect of the alanine mutations of the single residues of the region encompassing Pro^{1974} to Val^{1980} on the Nedd4-2-mediated decrease of $Na_v1.5$ I_{Na}. Nedd4-2 robustly decreased I_{Na} mediated by WT $Na_v1.5$ channels (open bar). Control (Ctrl) corresponds to the normalized peak I_{Na} measured when Nedd4-2 was not transfected. Most mutant channels altered Nedd4-2 regulation, with the exception of the Ala mutation of Ser^{1976}. $n = 10–15$ cells from at least four independent experiments. $^{**}P < 0.01$ compared with WT $Na_v1.5$, i.e., open bar. n.s., nonsignificant.

nal segment of $Na_v1.5$ in a PY-motif-dependent manner (Fig. 3, *A* and *B*). We therefore tested whether these other Nedd4-like E3s may ubiquitinate $Na_v1.5$ as previously described for Nedd4-2 in HEK-293 cells (31). Figure 4*A* shows that, by using an anti-ubiquitin antibody, ubiquitination of immunoprecipitated $Na_v1.5$ from the total cellular pool was clearly apparent when Nedd4-2 was coexpressed. In contrast, a much weaker increase in the ubiquitin signal was observed using Nedd4-1 and WWP2, despite comparable expression levels. Consistent with our functional and binding experiments, $Na_v1.5$-Y1977A mutant channels were not ubiquitinated by any of the three tested E3s (Fig. 4*A*).

We also investigated whether these other ubiquitin ligases may downregulate $Na_v1.5$ currents. The results of transient $Na_v1.5$ and Nedd4-like protein coexpression experiments are presented in Fig. 4*B*. Despite levels of expression comparable to that of Nedd4-2, Nedd4-1 did not downregulate $Na_v1.5$ I_{Na}, whereas WWP2 decreased I_{Na} by ~30%. These results suggest that either differences in binding affinity (e.g., between Nedd4-2 WW4 and Nedd4-1 WW4 domains; Table 1) or factors other than binding to the PY motif are responsible for the disparity in the efficiency with which Nedd4-like enzymes mediate ubiquitination and downregulation of $Na_v1.5$. These findings raise the possibility that, assuming proper cellular localization in cardiac cells, Nedd4-1 and WWP2 could antagonize Nedd4-2 action. This hypothesis was tested by cotransfecting, with $Na_v1.5$ and Nedd4-2, a twofold excess of cDNA encoding either Nedd4-1 or WWP2 compared with that of Nedd4-2. Western blots shown in Fig. 4*E* illustrate that, with this protocol, we approximately doubled the amount of Nedd4-1 and WWP2 compared with the conditions shown in Fig. 4, *B* and *C*. Under these conditions, WWP2 partially inhibited the Nedd4-2-dependent downregulation of $Na_v1.5$ I_{Na} (Fig. 4*D*). This effect was not mediated by a WWP2-dependent downregulation of Nedd4-2, because Nedd4-2 levels did not change upon cotransfection of the other E3 (Fig. 4*E*). In contrast, cotransfected Nedd4-1 did not interfere with Nedd4-2-dependent I_{Na} downregulation (Fig. 4*D*), despite its capacity to bind at the $Na_v1.5$ COOH terminus as shown in Fig. 3*A*.

Cellular mechanisms of Nedd4-2-mediated downregulation of $Na_v1.5$ I_{Na}. *Xenopus* Nedd4, the homolog of human Nedd4-2 (14), has been shown to decrease the cell membrane density of ENaC expressed in *Xenopus* oocytes (2). In the case of $Na_v1.5$, it was previously shown that the biophysical properties of

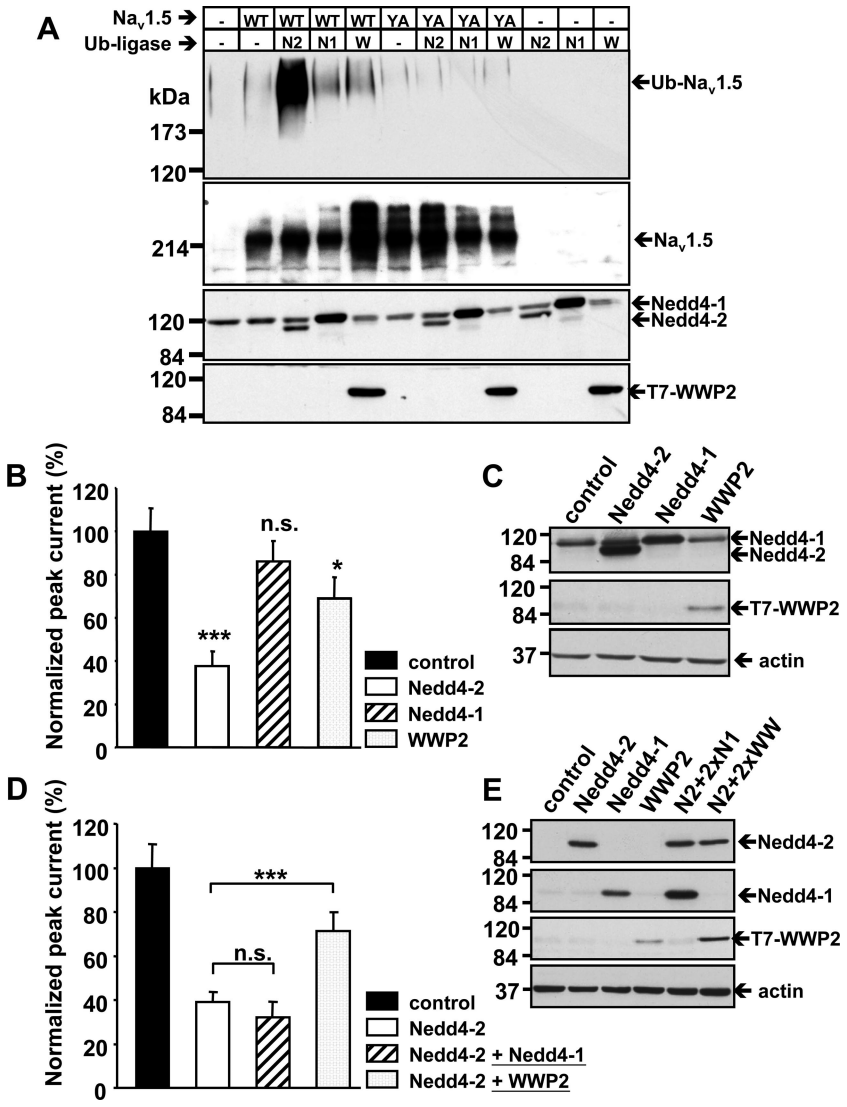
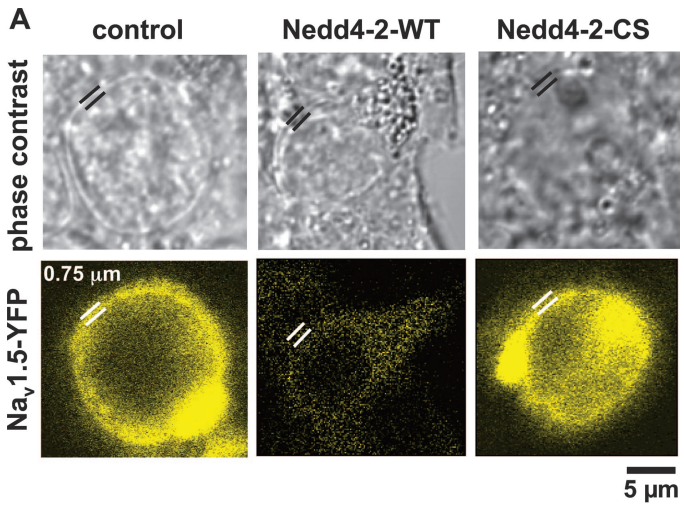


Fig. 4. Ubiquitination and regulation of $Na_v1.5$ I_{Na} by Nedd4-1, Nedd4-2, and WWP2, and competition between Nedd4-2 and the other Nedd4-like E3s. *A*: E3-ligase-dependent ubiquitination of $Na_v1.5$ was tested in HEK-293 cells transiently coexpressing $Na_v1.5$ WT and with the YA mutation (as indicated) with the three ubiquitin ligases Nedd4-2 (N2), Nedd4-1 (N1), and WWP2 (W). $Na_v1.5$ were immunoprecipitated using an isoform-specific antibody, and the precipitated fractions were examined using Western blot analysis with an anti-ubiquitin antibody (FK2). A robust increase in $Na_v1.5$ ubiquitination was observed only with Nedd4-2. This effect was not observed with the YA-mutated channel. The three *bottom* images show the levels of expression of $Na_v1.5$ and the three ubiquitin ligases using SP19 (pan-anti-Na_v) antibodies, antibodies cross-reacting with Nedd4-1 and Nedd4-2, and anti-T7, respectively. *B*: HEK-293 cells were transiently transfected with $Na_v1.5$ and the different Nedd4-like proteins. WWP2 decreased I_{Na} significantly by ~30%. In contrast, Nedd4-1 did not reduce I_{Na}; $n = 10$–30 cells from five independent experiments. $^*P < 0.05$, $^{***}P < 0.001$ compared with control. *C*: control Western blot analysis of protein expression of the three ubiquitin ligases tested in the experiments shown in *B*. The membrane shown in the *top* image was blotted using an antibody cross-reacting with Nedd4-2 and Nedd4-1 (14). *D*: WWP2 coexpression competed with Nedd4-2 downregulation of I_{Na}. HEK-293 cells were transiently transfected as in *B*, but in this experiment, Nedd4-2 was cotransfected with either Nedd4-1 or WWP2 (at double the cDNA amount) as shown in *E*. $n = 10$–30 cells from five independent experiments. $^{***}P < 0.001$. *E*: control Western blot analysis of the protein expression of the three ubiquitin ligases transfected in the experiment shown in *D*. *Lanes Nedd4-1* and *WWP2* are from the transfections shown in *B* and performed to show the increased (approximately double) expression of Nedd4-1 (*lane N2+2xN1*) and WWP2 (*lane N2+2xWW*) when the doubled amount of cDNA was used. *C* and *E*: protein loading was controlled by anti-actin immunoblotting.

$Na_v1.5$ remaining at the cell surface were not altered upon Nedd4-2 coexpression and that the peripheral localization of $Na_v1.5$ in HEK-293 cells was lost in the presence of Nedd4-2 (31). Together, these findings suggested that the Nedd4-2-dependent reduction in $Na_v1.5$ I_{Na} is caused by a decreased density of the channels at the cell membrane. Figure 5*A* shows the difference in cellular localization of a YFP-tagged form of $Na_v1.5$ with confocal microscopic imaging of transiently transfected HEK-293 cells with or without Nedd4-2-WT. Upon transfection of Nedd4-2-CS, the $Na_v1.5$ cellular localization was similar to that observed under control conditions (Fig. 5*A*). Quantification of the fluorescence intensity in a region of 0.75 μm encompassing the cell-cell contact region shows a decrease of ~80% (Fig. 5*B*) in Nedd4-2-WT-cotransfected cells. No significant decrease was observed with Nedd4-2-CS. These data strongly suggest that the observed ~80% reduction of I_{Na} (Fig. 3C) is caused by a reduced density of $Na_v1.5$ at the cell surface.



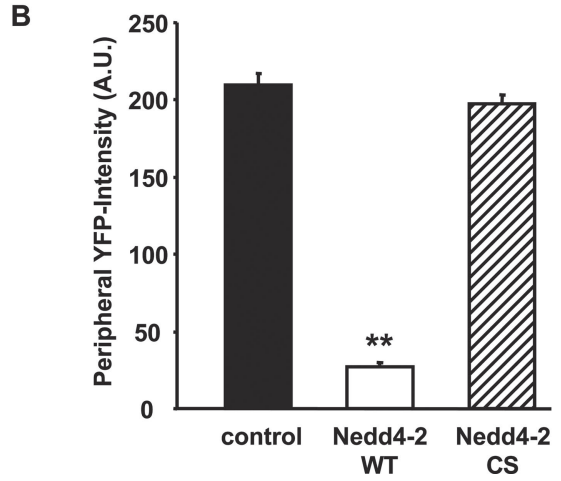


Fig. 5. $Na_v1.5$ cellular redistribution upon Nedd4-2-WT coexpression. *A*: representative HEK-293 cells transiently expressing $Na_v1.5$-YFP with or without Nedd4-2-WT or CS-mutant imaged by confocal microscopy (*bottom*). *Top*: corresponding phase-contrast images. Nedd4-2-WT, but not Nedd4-2-CS, clearly redistributed $Na_v1.5$ from a peripheral localization to a more homogeneous distribution. Fluorescence intensity was measured in a 0.75-μm-wide region encompassing the cell membrane (schematically indicated with the two black and two white lines). *B*: peripheral fluorescence intensity quantification as described in *A* for HEK-293 cells expressing $Na_v1.5$-YFP cotransfected with empty vector (control), Nedd4-2-WT, and Nedd4-2-CS. $n = 8$. $^{**}P < 0.01$. A.U., arbitrary units.

Finally, the question of whether Nedd4-2 regulates the $Na_v1.5$ secretory or internalization and/or endocytotic pathways was addressed using a HEK-293 cell line stably expressing this channel (23). Figure 6*A* shows the results obtained when measuring the $Na_v1.5$ I_{Na} density at different time points after addition of 50 ng/ml brefeldin A (BFA), a fungal metabolite that inhibits ADP ribosylating factor-mediated vesicular transport and disrupts the Golgi apparatus (19). Using BFA, one can expect to block the trafficking of newly synthesized channels toward the cell surface and consequently assess the half-life of the pool of channels at the membrane. In parallel, these cells were transiently transfected with either empty plasmid (control) or Nedd4-2-WT 24 h before addition of BFA or vehicle. Upon BFA treatment, I_{Na} decreased gradually toward values close to zero, with a half-time of 13.3 h as fitted using a monoexponential function on the averaged data points (Fig. 6, *A* and *B*, bold curves). The time course of I_{Na} starting 24 h after Nedd4-2 transfection was biphasic. We first observed a decay of $Na_v1.5$ I_{Na}, with a minimum observed after ~44 h posttransfection ($t = 20$ h in Fig. 6*A*, thin biphasic curve). Afterward, I_{Na} recovered gradually, reaching values close to those of the control transfected cells (~72 h after transfection). This phenomenon is most probably caused by the parallel increase and decrease in Nedd4-2 expression over time as revealed in Western blot experiments (Fig. 6, *C* and *D*). When BFA was added to the Nedd4-2-transfected cells, I_{Na} decayed more rapidly than in control transfected cells treated only with BFA. Monoexponential fit (Fig. 6, *A* and *B*, dotted curves) of the averaged data yielded a decay half-time of 5.8 h. These findings are consistent with a model in which Nedd4-2 accelerates the rate of internalization of $Na_v1.5$ channels from the cell surface. A more complex model in which Nedd4-2 could also act simultaneously on the secretory pathway cannot be excluded on the basis of these data. On the other hand, it cannot be excluded that BFA disrupts unknown factors involved in stabilizing $Na_v1.5$ at the cell membrane.

DISCUSSION

Two recent studies have shown that Na_v bearing a PY motif in the COOH termini are negatively regulated by protein-ubiquitin ligases of the Nedd4/Nedd4-like family (5, 31). In the present report, we describe novel data complementing and expanding these findings. First, using both brain lysates and a mammalian expression system, we have shown that the PY motifs of $Na_v1.5$ and the neuronal isoforms $Na_v1.2$ and $Na_v1.3$ interacted with brain Nedd4-2 and that these three channels are downregulated by this enzyme. Second, we have demonstrated that not only Nedd4-2 but also Nedd4-1 and WWP2 bind to the extended PY motif of $Na_v1.5$ and that Nedd4-1 and WWP2 only weakly ubiquitinate $Na_v1.5$. Third, we have provided evidence that WWP2 may compete with Nedd4-2. Fourth, we have shown that Nedd4-2 decreases Na_v-mediated currents via a reduction of the number of channels at the cell surface by increasing their internalization rate.

Regulation of Na_v channels by Nedd4-like E3s can be extended to neuronal channels. The conservation of the extended PY motif with the consensus sequence (L/P)PxYxx(V/L) in the

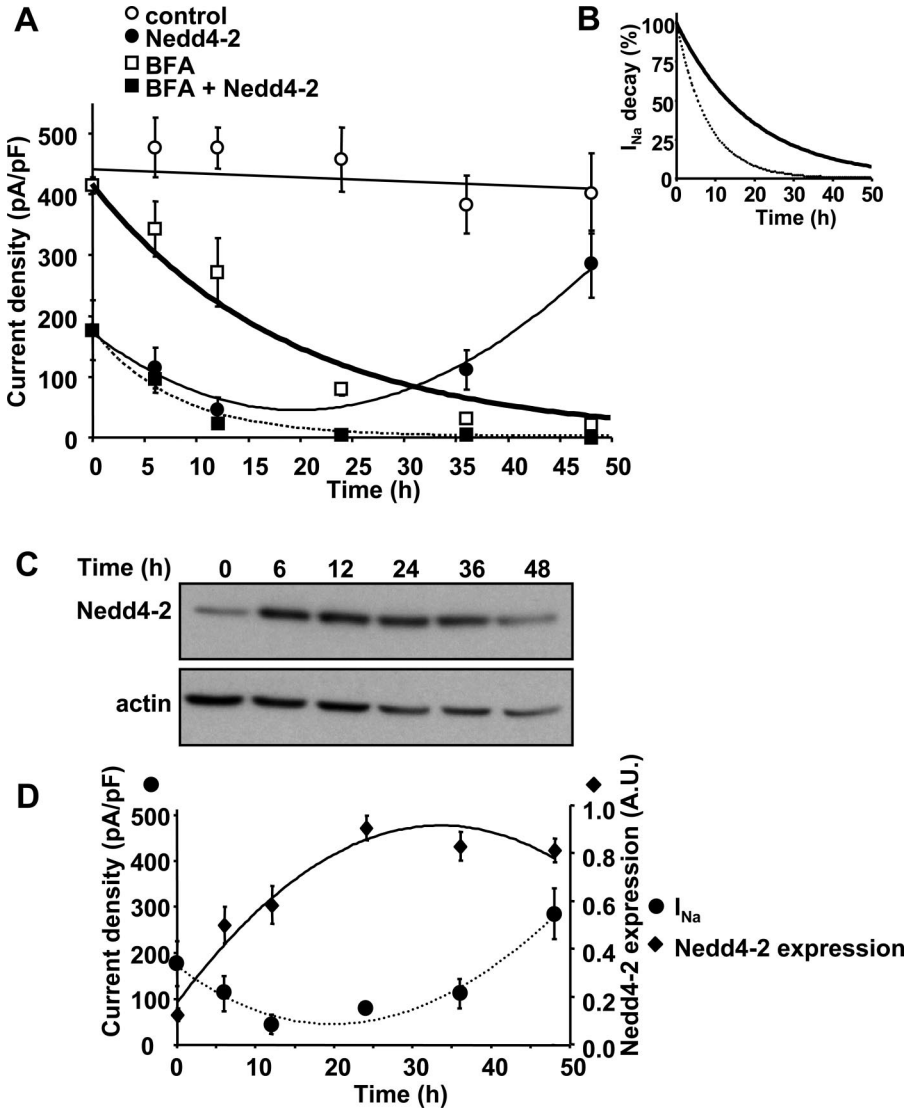


Fig. 6. Modulation of $Na_v1.5$ internalization rate by Nedd4-2. *A*: time course of peak current densities of HEK-293 cells stably expressing $Na_v1.5$ in four experimental conditions: control (○), cells treated with 50 ng/ml brefeldin A (BFA) (□), transiently transfected 24 h before addition of BFA ($t = 0$) with Nedd4-2 (●), and transiently transfected 24 h earlier with Nedd4-2 and treated with BFA ($t = 0$) (■). The bold and dotted curves are monoexponential fits of the I_{Na} decays after BFA treatment of control (bold curve) and Nedd4-2-transfected cells (dotted curve). $n = 4–7$ cells per condition. The thin curves were obtained via interpolation of the data points (see text for details). *B*: normalization of the fitted I_{Na} decays shown in *A* after BFA suggests that Nedd4-2 increases the internalization rate of $Na_v1.5$ (bold line, BFA alone; dotted line, BFA+Nedd4-2). *C* and *D*: as shown in *A* (●) under control conditions (i.e., without BFA treatment), the effect of Nedd4-2 transient transfection on I_{Na} was biphasic. Quantification of Nedd4-2 expression at the different time points was performed on three Western blots (*C*, *top*) by normalizing the intensities of the Nedd4-2 bands to the actin bands (*C*, *bottom*). The time course of Nedd4-2 expression (*D*, ◆) correlated with the observed negative but transient effect of Nedd4-2 on the $Na_v1.5$-mediated currents (*D*, ●), suggesting that this biphasic phenomenon is caused by the transient expression of Nedd4-2.

COOH-terminal region of seven Na_v members is a striking feature. The sequences are identical (Fig. 1), with the exception of leucine instead of the first proline in $Na_v1.6$. In fact, it has been reported that the first proline in PY motifs can be substituted with leucine without loss of binding affinity to WW domains (17). The results of the present work, along with those of Fotia et al. (5), clearly have shown that not only the cardiac $Na_v1.5$ channels but also probably most of the neuronal channels can be regulated by Nedd4-like proteins. Our observations indicate that common features allowing for ubiquitination and internalization, other than the PY motif, are also shared by these different channels. At present, very little is known about the molecular and cellular mechanisms involved in the regulation of the membrane density of Na_v channels in excitable cells. As exemplified by a number of human diseases caused by sometime subtle gain- or loss-of-function mutations in Na_v genes, normal cellular function does not tolerate variation in the number of functional Na_v channels at the cell surface. Thus far, no mutation in the PY motif of any Na_v genes has been described. Besides the probable role of the β-subunits in increasing the channel density at the cell membrane (12), the present work further supports the role played by Nedd4-like E3s in regulating the density of Na_v channels.

The ubiquitin system, and in particular this class of E3s, has been shown to play an important role in the nervous system (8). Nedd4-1, the first identified member of this family of nine genes (11), is strongly expressed in the embryonic mouse brain (20, 21), and its *Drosophila* homolog, dNedd4, is an important factor in axonal guidance during brain development by targeting the protein Commissureless (25). Besides neurological diseases such as genetic forms of epilepsy caused by mutations in $Na_v1.1$ and $Na_v1.2$ genes (22), Na_v most probably play a central role in acquired disorders such as chronic pain and multiple sclerosis (32). It can be speculated that the Nedd4-like-dependent regulation or dysregulation of Na_vs may represent a mechanism involved in normal and pathological states.

Molecular determinants of the interaction between Na_v and Nedd4-like proteins. The structural factors important for the interaction of the β-ENaC extended PY motif, PPnYdsL, with the WW domains of Nedd4-1 and Nedd4-2 have been studied in detail (9, 15, 16). The WW domains form a hydrophobic binding surface comprising two stabilizing surfaces promoting

the interaction with the PY-motif ligand. An XP groove surface interacts with the polyproline type II helix, and a second surface interacts with the Tyr of the PY motif. These studies also provided strong evidence that the residues following the traditional PPxY motif make a sharp helical turn, allowing the methyl group of the Tyr+3 aliphatic residue (Leu^{621} in β-ENaC and Val^{1980} in $Na_v1.5$) to interact with the WW domain, thus providing additional binding energy. Overall, the binding and functional data presented in the present report as well as in a previous study conducted in our laboratory (31) are in agreement with a similar type of interaction that takes place between the extended Na_v PY motif and the WW domains of Nedd4-2 or other Nedd4-like proteins. The measured affinity of $Na_v1.5$ PY-motif peptide with WW domains was highest in the WW4 of Nedd4-2, a finding that contradicts that of Fotia et al. (5), who found that the interaction was stronger with the WW3 of Nedd4-2, although K_d measurements were not provided in their study. This inconsistency may be caused by the fact that the binding assays were different (tryptophan fluorescence measurement vs. Far Western blot analysis). The absolute value of ~55 μM represents a moderate-affinity interaction. However, as suggested by the specificity of our findings, it is possible that in the cellular context, other factors such as partner proteins may increase the strength of this interaction. Nevertheless, this interaction seems to be weak and transient as exemplified by our failure to coimmunoprecipitate the two full-length proteins from cell lysates (van Bemmelen MX and Abriel H, unpublished data).

The results of the $Na_v1.5$ PY-motif alanine scan showed a slight discrepancy between the binding (pull-down) experiments and the Nedd4-2-dependent downregulation of the $Na_v1.5$ currents. Single replacements of the Tyr+1 to Tyr+3 residues (i.e., Asp-Ser-Val) with Ala did not interfere with the capacity of the GST fusion proteins to interact with the tested E3s. In contrast, the channels bearing the same mutations expressed in HEK-293 cells were less efficiently regulated by Nedd4-2 compared with WT $Na_v1.5$ (Fig. 3*C*). This observation suggests either that the pull-down approach used does not have enough resolution to discriminate low-affinity differences or that the functional consequences of the interaction between Nedd4-2 and $Na_v1.5$ may be dependent on the integrity of this sequence in the cellular context.

Surprisingly, we did not observe any saturable binding to any WW domains using the hERG PY-motif peptide, despite the fact that its sequence (PPaYsaV) corresponds well to the predicted structural requirements. One possible difference may be the Tyr+1 residue that is occupied by a negatively charged residue in the PY-motif sequences with the highest affinities (β-ENaC and Na_v). Interestingly, in a recent large-scale screen of WW domains binding peptides (10), peptides with negative charges in positions Tyr+1 and +2 were shown to be preferred. Further experiments are necessary to elucidate the mechanisms underlying this observation.

Diversity in the E3s and specificity in the effects. In the current study, we tested three of the nine known E3s present in the human genome, i.e., Nedd4-1, Nedd4-2, and WWP2. Analogously to the work of Fotia et al. (5), who tested Nedd4-1 and Nedd4-2, we observed striking differences in the capacity of these ligases to ubiquitinate and regulate Na_v channels. Indeed, despite the fact that Nedd4-1 and WWP2 were able to bind well to the PY motif of $Na_v1.5$, their efficacy in ubiquitinating and downregulating $Na_v1.5$ was very weak compared with Nedd4-2. Furthermore, we observed that, when coexpressed with Nedd4-2, WWP2 was competing with the Nedd4-2-dependent downregulation of $Na_v1.5$ currents. The mechanism of such competition, described in the present report for the first time, is not clear. It seems, however, that it cannot be based solely on the fact that both E3s compete for the same binding site, i.e., PY motif of $Na_v1.5$, because such competition was not observed with Nedd4-1 (Fig. 4*D*). It could be that unknown cellular factors are necessary for both Nedd4-2 and WWP2 and that coexpression of WWP2 may reduce the availability of these factors for Nedd4-2. Together, these results illustrate the potential complexity of these regulatory mechanisms, and, as a consequence, further investigations are needed to answer these questions.

Cellular mechanisms of Nedd4-2 regulation. In this study, we have provided direct experimental evidence for a role of Nedd4-2 in internalization of cell membrane ion channels in mammalian cells. Coexpression of Nedd4-2 with $Na_v1.5$ leads to a strong redistribution of $Na_v1.5$ from the cell surface to undefined intracellular compartments. In agreement with these findings, the experiments performed in the presence of BFA indicated that Nedd4-2 increased the $Na_v1.5$ disappearance rate. Mutations of the PY motifs of ENaC β- or γ-subunits found in humans lead to a hereditary form of hypertension known as Liddle's syndrome. It has been demonstrated clearly that ENaC in patients with Liddle's syndrome are less efficiently regulated by Nedd4-like proteins and also that they accumulate at the cell surface of *Xenopus* oocytes (2, 18) and renal cells (3). When BFA was used to block the cellular secretory pathway, Nedd4-2 more than doubled the rate at which the I_{Na} decreased. This observation further supports the model proposing that Nedd4-2 directly ubiquitinates $Na_v1.5$ as presented in Fig. 4*A* and that this ubiquitination enhances the rate of endocytosis of the channels. However, thus far, the molecular mechanisms underlying this phenomenon are poorly understood, and analogously to recent reports (24, 29), ubiquitination of $Na_v1.5$ may be important for proper sorting at the early endosomal stage rather than for the internalization process. Increasing the sorting of $Na_v1.5$ toward early endosomes and/or decreasing the putative recycling of channels to the membrane could result in fewer channels at the cell surface.

Physiological relevance. At this stage, the relevance of these findings in normal and abnormal cellular physiology is only speculative. Several observations, however, point to a physiological role of this proposed mode of regulation of Na_v channels. The extended PY motif found in Na_v channels is very similar to motifs found in the different subunits of ENaC, most particularly in the β-subunit. This motif is very well conserved among the different members of the family of Na_vs, despite the fact that the distal parts of the COOH termini of these channels are variable. Moreover, ubiquitinated forms of $Na_v1.5$ have been found in cardiac tissues, suggesting a physiological role for this type of protein posttranslational modification (31). Finally, researchers in this area face a rather complex situation, because there is, on the one hand, a family with seven channels containing a PY motif, and on the other hand, a family of nine Nedd4/Nedd4-like ubiquitin-protein ligases that are widely expressed in excitable cells. In theory, this allows the possibility of a very large number of complex ways to regulate Na_v density at the cell membrane. Most likely, only systematic and

large-scale approaches using small interfering RNA silencing cellular models, total knockout mice, and tissue-specific knockout mice may provide information that will help to address the issue of complexity.

ACKNOWLEDGMENTS

We thank K. Geering, M. Harris, J.-D. Horisberger, S. Kellenberger, and J. Loffing for critically reading the manuscript. The excellent technical help of X. Ding in generating mutant constructs is acknowledged. We thank the imaging facility team of the Faculty of Biology and Medicine for help in using the confocal microscope. All patch-clamp experiments were performed in the Department of Physiology of the University of Lausanne with the help of P. Kucera.

GRANTS

This work was supported by Swiss National Science Foundation Grants 632-66149.01 (SNF professorship to H. Abriel) and 3100A0-103779 (to O. Staub), the Nicod-Botnar Foundation and the Fondation Vaudoise de Cardiologie (to H. Abriel), and the Canadian Institutes of Health Research and the Canadian Cystic Fibrosis Foundation (to D. Rotin). We also thank Y. J. Paternot for generous financial support.

REFERENCES

1. **Abriel H, Kamynina E, Horisberger JD, and Staub O.** Regulation of the cardiac voltage-gated Na^+ channel (H1) by the ubiquitin-protein ligase Nedd4. *FEBS Lett* 466: 377–380, 2000.
2. **Abriel H, Loffing J, Rebhun JF, Pratt JH, Schild L, Horisberger JD, Rotin D, and Staub O.** Defective regulation of the epithelial Na^+ channel by Nedd4 in Liddle's syndrome. *J Clin Invest* 103: 667–673, 1999.
3. **Auberson M, Hoffmann-Pochon N, Vandewalle A, Kellenberger S, and Schild L.** Epithelial Na^+ channel mutants causing Liddle's syndrome retain ability to respond to aldosterone and vasopressin. *Am J Physiol Renal Physiol* 285: F459–F471, 2003.
4. **Catterall WA.** From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* 26: 13–25, 2000.
5. **Fotia AB, Ekberg J, Adams DJ, Cook DI, Poronnik P, and Kumar S.** Regulation of neuronal voltage-gated sodium channels by the ubiquitin-protein ligases Nedd4 and Nedd4-2. *J Biol Chem* 279: 28930–28935, 2004.
6. **Goldin AL.** Resurgence of sodium channel research. *Annu Rev Physiol* 63: 871–894, 2001.
7. **Harvey KF and Kumar S.** Nedd4-like proteins: an emerging family of ubiquitin-protein ligases implicated in diverse cellular functions. *Trends Cell Biol* 9: 166–169, 1999.
8. **Hegde AN and DiAntonio A.** Ubiquitin and the synapse. *Nat Rev Neurosci* 3: 854–861, 2002.
9. **Henry PC, Kanelis V, O'Brien MC, Kim B, Gautschi I, Forman-Kay J, Schild L, and Rotin D.** Affinity and specificity of interactions between Nedd4 isoforms and the epithelial Na^+ channel. *J Biol Chem* 278: 20019–20028, 2003.
10. **Hu H, Columbus J, Zhang Y, Wu D, Lian L, Yang S, Goodwin J, Luczak C, Carter M, Chen L, James M, Davis R, Sudol M, Rodwell J, and Herrero JJ.** A map of WW domain family interactions. *Proteomics* 4: 643–655, 2004.
11. **Ingham RJ, Gish G, and Pawson T.** The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. *Oncogene* 23: 1972–1984, 2004.
12. **Isom LL.** Sodium channel β subunits: anything but auxiliary. *Neuroscientist* 7: 42–54, 2001.
13. **Itani OA, Campbell JR, Herrero J, Snyder PM, and Thomas CP.** Alternate promoters and variable splicing lead to hNedd4-2 isoforms with a C2 domain and varying number of WW domains. *Am J Physiol Renal Physiol* 285: F916–F929, 2003.
14. **Kamynina E, Tauxe C, and Staub O.** Distinct characteristics of two human Nedd4 proteins with respect to epithelial Na^+ channel regulation. *Am J Physiol Renal Physiol* 281: F469–F477, 2001.
15. **Kanelis V, Farrow NA, Kay LE, Rotin D, and Forman-Kay JD.** NMR studies of tandem WW domains of Nedd4 in complex with a PY motif-containing region of the epithelial sodium channel. *Biochem Cell Biol* 76: 341–350, 1998.
16. **Kanelis V, Rotin D, and Forman-Kay JD.** Solution structure of a Nedd4 WW domain-ENaC peptide complex. *Nat Struct Biol* 8: 407–412, 2001.
17. **Kasanov J, Pirozzi G, Uveges AJ, and Kay BK.** Characterizing class I WW domains defines key specificity determinants and generates mutant domains with novel specificities. *Chem Biol* 8: 231–241, 2001.
18. **Kellenberger S, Gautschi I, Rossier BC, and Schild L.** Mutations causing Liddle syndrome reduce sodium-dependent downregulation of the epithelial sodium channel in the *Xenopus* oocyte expression system. *J Clin Invest* 101: 2741–2750, 1998.
19. **Klausner RD, Donaldson JG, and Lippincott-Schwartz J.** Brefeldin A: insights into the control of membrane traffic and organelle structure. *J Cell Biol* 116: 1071–1080, 1992.
20. **Kumar S, Harvey KF, Kinoshita M, Copeland NG, Noda M, and Jenkins NA.** cDNA cloning, expression analysis, and mapping of the mouse Nedd4 gene. *Genomics* 40: 435–443, 1997.
21. **Kumar S, Tomooka Y, and Noda M.** Identification of a set of genes with developmentally down-regulated expression in the mouse brain. *Biochem Biophys Res Commun* 185: 1155–1161, 1992.
22. **Lehmann-Horn F and Jurkat-Rott K.** Nanotechnology for neuronal ion channels. *J Neurol Neurosurg Psychiatry* 74: 1466–1475, 2003.
23. **Malhotra JD, Chen C, Rivolta I, Abriel H, Malhotra R, Mattei LN, Brosius FC, Kass RS, and Isom LL.** Characterization of sodium channel α- and β-subunits in rat and mouse cardiac myocytes. *Circulation* 103: 1303–1310, 2001.
24. **Marchese A, Raiborg C, Santini F, Keen JH, Stenmark H, and Benovic JL.** The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Dev Cell* 5: 709–722, 2003.
25. **Myat A, Henry P, McCabe V, Flintoft L, Rotin D, and Tear G.** Drosophila Nedd4, a ubiquitin ligase, is recruited by Commissureless to control cell surface levels of the roundabout receptor. *Neuron* 35: 447–459, 2002.
26. **Pace CN, Vajdos F, Fee L, Grimsley G, and Gray T.** How to measure and predict the molar absorption coefficient of a protein. *Protein Sci* 4: 2411–2423, 1995.
27. **Rotin D, Staub O, and Haguenauer-Tsapis R.** Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. *J Membr Biol* 176: 1–17, 2000.
28. **Schwake M, Friedrich T, and Jentsch TJ.** An internalization signal in ClC-5, an endosomal Cl^- channel mutated in Dent's disease. *J Biol Chem* 276: 12049–12054, 2001.
29. **Sharma M, Pampinella F, Nemes C, Benharouga M, So J, Du K, Bache KG, Papsin B, Zerangue N, Stenmark H, and Lukacs GL.** Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. *J Cell Biol* 164: 923–933, 2004.
30. **Staub O, Dho S, Henry P, Correa J, Ishikawa T, McGlade J, and Rotin D.** WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na^+ channel deleted in Liddle's syndrome. *EMBO J* 15: 2371–2380, 1996.
31. **Van Bemmelen MX, Rougier JS, Gavillet B, Apothéloz F, Daidie D, Tateyama M, Rivolta I, Thomas MA, Kass RS, Staub O, and Abriel H.** Cardiac voltage-gated sodium channel Nav1.5 is regulated by Nedd4-2 mediated ubiquitination. *Circ Res* 95: 284–291, 2004.
32. **Waxman SG.** Acquired channelopathies in nerve injury and MS. *Neurology* 56: 1621–1627, 2001.
33. **Zimmer T, Biskup C, Dugarmaa S, Vogel F, Steinbis M, Bohle T, Wu YS, Dumaine R, and Benndorf K.** Functional expression of GFP-linked human heart sodium channel (hH1) and subcellular localization of the α-subunit in HEK293 cells and dog cardiac myocytes. *J Membr Biol* 186: 1–12, 2002.