

# Regulation of the Voltage-gated K<sup>+</sup> Channels KCNQ2/3 and KCNQ3/5 by Ubiquitination

## NOVEL ROLE FOR Nedd4-2\*

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The muscarine-sensitive K<sup>+</sup> current (M-current) stabilizes the resting membrane potential in neurons, thus limiting neuronal excitability. The M-current is mediated by heteromeric channels consisting of KCNQ3 subunits in association with either KCNQ2 or KCNQ5 subunits. The role of KCNQ2/3/5 in the regulation of neuronal excitability is well established; however, little is known about the mechanisms that regulate the cell surface expression of these channels. Ubiquitination by the Nedd4/Nedd4-2 ubiquitin ligases is known to regulate a number of membrane ion channels and transporters. In this study, we investigated whether Nedd4/Nedd4-2 could regulate KCNQ2/3/5 channels. We found that the amplitude of the K<sup>+</sup> currents mediated by KCNQ2/3 and KCNQ3/5 were reduced by Nedd4-2 (but not Nedd4) in a *Xenopus* oocyte expression system. Deletion experiments showed that the C-terminal region of the KCNQ3 subunit is required for the Nedd4-2-mediated regulation of the heteromeric channels. Glutathione S-transferase fusion pull-downs and co-immunoprecipitations demonstrated a direct interaction between KCNQ2/3 and Nedd4-2. Furthermore, Nedd4-2 could ubiquitinate KCNQ2/3 in transfected cells. Taken together, these data suggest that Nedd4-2 is potentially an important regulator of M-current activity in the nervous system.

The muscarine-sensitive K<sup>+</sup> current (M-current)<sup>4</sup> is a low threshold, non-inactivating K<sup>+</sup> current that was first identified in sympathetic neurons (1) and is now known to be an impor-

tant regulator of the excitability of many neuronal cell types in the central and peripheral nervous systems. The characteristic voltage and time dependence of the M-current leads to a “clamping” of the resting membrane potential upon activation of the current. The M-current therefore exerts a stabilizing effect on the resting membrane potential and attenuates neuronal excitability, thus limiting repetitive firing of neurons (2, 3). The M-current is mediated by members of the K<sub>v</sub>7 family, which form a heterotetrameric channel consisting of KCNQ3 subunits associated with either KCNQ2 or KCNQ5 subunits, all containing six transmembrane domains (S1–S6), a short intracellular N-terminal segment, and a large intracellular C-terminal region (4–8). Mutations in KCNQ2 and KCNQ3 are associated with epilepsy, in particular, benign neonatal familial seizures, and suppression of the M-current in conditional transgenic mice has been reported to cause spontaneous seizures (7, 9).

At the single channel level, the M-current has been reported to be regulated by several receptor-mediated pathways and changes in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) (reviewed in Refs. 2 and 10); however, little is known about the factors that determine the cell surface density of the channels that underlie this current. One recently characterized mechanism for the regulation of cell surface levels of membrane proteins is ubiquitination by the Nedd4/Nedd4-2 ubiquitin ligases, which results in the removal of the protein from the membrane (reviewed in Refs. 11 and 12). This mechanism was originally identified in the regulation of the epithelial Na<sup>+</sup> channel ENaC (13); however, Nedd4/4-2 are now known to regulate a diverse number of ion channels, receptors, and transporters in a variety of tissues. In neurons, these include voltage-gated Na<sup>+</sup> channels (14), the voltage-gated K<sup>+</sup> channels, the renal outer medullary K<sup>+</sup> channel (15) and K<sub>v</sub>1.3 (16), the Cl<sup>-</sup> channel ClC-2 (17), the glutamate transporter EEAT (18), and the dopamine transporter (19). Nedd4/4-2 contains a HECT (homologous to E6-AP C terminus) ubiquitin ligase domain, an N-terminal C2 domain involved in membrane localization, and four WW (Trp-Trp) domains. These WW domains typically bind to PY motifs (PPXY) in the C terminus of target proteins (20–22); however, Nedd4/Nedd4-2 can also regulate proteins that lack these motifs (16, 18). The C termini of both KCNQ3 and KCNQ2 contain atypical PY motifs PPX-PPY and XPXPPY. We have recently shown that the PPXPPY

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<sup>4</sup> The abbreviations used are: M-current, muscarine-sensitive K<sup>+</sup> current; KCNQ3Δ, truncated C terminus of KCNQ3; Nedd4, neural precursor cell-expressed, developmentally down-regulated 4; wt, wild-type; GST, glutathione S-transferase; HA, hemagglutinin; HEK, human embryonic kidney; PBS, phosphate-buffered saline.

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sequence in the Cl<sup>-</sup> channel ClC-5 binds Nedd4-2 and that this interaction controls cell surface levels of ClC-5 in the proximal tubule cells (23). Thus, the presence of these motifs in the C terminus of KCNQ2 and KCNQ3 suggests a potential role for Nedd4/Nedd4-2 in the regulation of these channels, and the current study was undertaken to explore this possibility.

### EXPERIMENTAL PROCEDURES

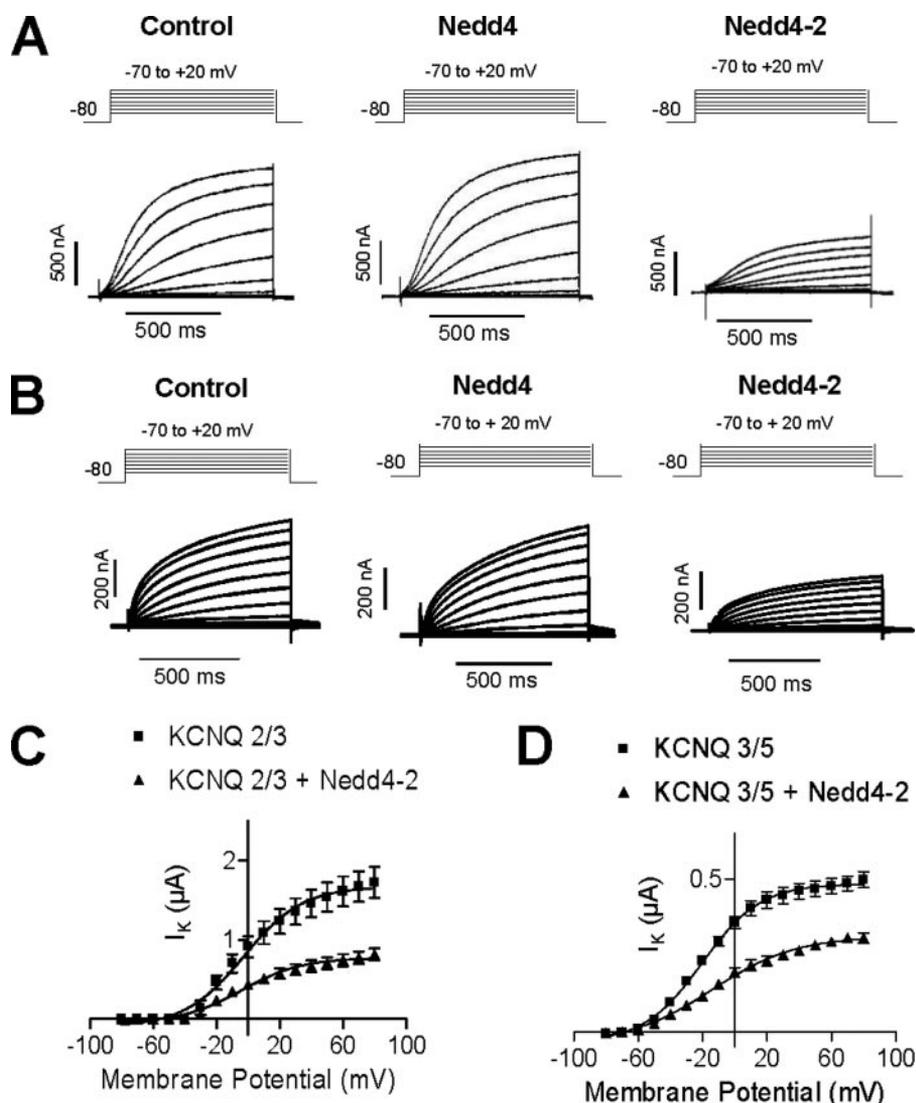
**Plasmids and Expression Constructs**—Rat KCNQ2 and KCNQ3 were received from Dr. David McKinnon, State University of New York, Stony Brook, NY. Rat KCNQ5 was a gift from Dr. Thomas J. Jentsch, Hamburg University, Zentrum für Molekulare Neurobiologie, Hamburg, Germany. A limited deletion of the C-terminal region of the KCNQ3 subunit was carried out using the restriction enzyme StuI (New England Biolabs) to delete approximately the last two-thirds (~1000 bp) of the C terminus. The PY motif of KCNQ3 (PPDPPY) was mutated by exchanging tyrosine against alanine at position 698 using standard PCR-based, site-directed mutagenesis with *Pfu* Turbo polymerase (Stratagene) to generate KCNQ3[Y698A]. Capped RNA transcripts encoding full-length rat KCNQ2, wild-type (wt) and PY mutant rat KCNQ3, rat KCNQ5, human Nedd4, human Nedd4-2, and a ligase-deficient mutant of Nedd4-2 (Nedd4-2[C922S]) were synthesized using an mMESSAGE mMACHINE *in vitro* transcription kit (Ambion). The coding region of rat KCNQ2 was amplified by PCR with an N terminus Myc tag and cloned into the HindIII and XhoI sites of pcDNA3.1 (Invitrogen) to generate pcKCNQ2-Myc. pcKCNQ3-V5 was generated by PCR amplification of the rat KCNQ3 open reading frame with a V5 tag (GKPIPPLLGLDST) engineered at the N terminus and cloned into the HindIII and XhoI sites of pcDNA3.1. pcKCNQ3-mV5 (PY mutant) was similarly generated, except that the rat KCNQ3 open reading frame had a Y698A mutation introduced via site-directed mutagenesis. hNedd-1-Myc (24) was generated by PCR amplification of the open reading frame of human Nedd1 (GenBank<sup>TM</sup> accession number NM\_152905) and cloned into the BglII and XhoI sites of pCMV-Myc (Clontech). The generation of pcDNA3-Nedd4-2-FLAG and pcDNA3-Nedd4-2[C922S]-FLAG have been described previously (25, 26). Hemagglutinin (HA)-tagged ubiquitin (Ub-HA) was kindly provided by Dr. Dirk Bohmann, Aab Institute Biomedical Sciences, Rochester, NY.

**Electrophysiological Recording of K<sup>+</sup> Currents Mediated by KCNQ2/3 and KCNQ3/5 Expressed in Xenopus Oocytes**—*Xenopus laevis* stages V–VI oocytes were removed and treated with collagenase (Sigma type I) for defolliculation. Oocytes were injected with combinations of KCNQ2/3 (7.5 ng/oocyte) and KCNQ3/5 (7.5 ng/oocyte) alone and in combination with wt Nedd4-2 or Nedd4-2[C922S] (5 ng/oocyte). Similar results were obtained from oocytes injected with lower amounts of RNA for Nedd4-2. The oocytes were incubated at 18 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 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 K<sup>+</sup> 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 0.2–2.0 MΩ. All recordings were made at room temperature (20–23 °C) using ND96 solution. Using a GeneClamp 500B amplifier, a Digidata 1322A interface, and pCLAMP 8 software (Axon Instruments Inc, Union City, CA), data were low pass-filtered at 1 kHz, digitized at 10 kHz, and analyzed off-line. To generate outward K<sup>+</sup> currents, oocytes were voltage-clamped at a holding potential of –80 mV and depolarized to voltages between –70 mV and +80 mV in 10-mV increments for 1 s depending on the experiments. Unless otherwise indicated, statistical significance was analyzed using one-way analysis of variance with Tukey's multiple comparison test.

**Immunoprecipitations and Ubiquitination Assays**—HEK293T cells were transfected with expression constructs as indicated using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions for adherent cells. Cells were harvested 24 h post-transfection and lysed for 30 min at 4 °C in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and Complete Protease inhibitors (Roche Applied Science). Protein G-Sepharose (10 μl, GE Healthcare) was incubated with the cell lysate for 2 h at 4 °C. The precleared lysate was then incubated at 4 °C overnight with the appropriate antibody and protein G-Sepharose. Immunoprecipitates were washed twice in lysis buffer and once in phosphate-buffered saline (PBS), dissolved in 30 μl of protein loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 1% β-mercaptoethanol), subjected to 8–10% SDS-PAGE, and transferred to polyvinylidene difluoride membrane (PerkinElmer Life Sciences). The following primary antibodies were used: anti-Myc mouse monoclonal antibody (5 μg/ml, Roche Applied Science), anti-V5 rabbit affinity-purified antibody (1 μg/ml, Bethyl Laboratories, Inc.), anti-HA mouse monoclonal antibody (5 μg/ml, Roche Applied Science), and anti-FLAG mouse monoclonal antibody (4.3 μg/ml, Sigma). Alkaline phosphatase-conjugated anti-mouse and anti-rabbit IgG antibodies were used (1:2000, Chemicon), and horseradish peroxidase-conjugated anti-mouse (1:2000, GE Healthcare) were used as secondary antibodies. Detection of bound antibody was achieved using either ECF (Amersham Biosciences) and quantification with a Typhoon 9410 variable mode imager (Molecular Dynamics, Inc.) or the ECL Plus Western blotting detection kit (GE Healthcare) and exposure to Fuji medical x-ray Super Rx film.

**Immunofluorescence Analysis**—KCNQ2-Myc (2 μg), KCNQ3-V5 (2 μg), and Nedd4-2 (4 μg) were co-transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen). Cells were stained after 24 h as follows. Cells were washed in PBS, fixed in 4% paraformaldehyde/PBS for 10 min, and then permeabilized in 0.1% Triton X-100/PBS for 10 min. Cells were then blocked in 1% fetal calf serum/PBS for 30 min and stained with mouse anti-Myc (1:200) and rabbit anti-Nedd4-2 (1:200) for 2 h. For the secondary antibodies, anti-mouse Alexa Fluor 568 and anti-rabbit Alexa Fluor 488 (Molecular Probes), both at 1:1000, were added for 1 h before samples were mounted onto slides and imaged using a confocal microscope (Radiance 2100; Bio-Rad Laboratories).



**FIGURE 1. Nedd4-2 down-regulates the  $K^+$  currents mediated by KCNQ2/3 and KCNQ3/5 expressed in *Xenopus* oocytes.** *A*, representative family of depolarization-activated  $K^+$  currents recorded from *Xenopus* oocytes expressing the KCNQ2/3 heteromer in the absence and presence of Nedd4 or Nedd4-2. Oocytes were held at  $-80$  mV and depolarized to voltages ranging from  $-70$  to  $+20$  mV in  $10$ -mV increments for  $1$  s. *B*, representative family of depolarization-activated  $K^+$  currents mediated by the KCNQ3/5 heteromer alone and in the presence of Nedd4 or Nedd4-2. Voltage steps were applied from  $-70$  to  $+20$  mV in  $10$ -mV increments from a holding potential of  $-80$  mV. *C*, current-voltage relationships of KCNQ2/3 obtained in the absence and presence of Nedd4-2 expressed in oocytes. Data are expressed as mean  $\pm$  S.E. of 10 oocytes/group. *D*, current-voltage relationships of KCNQ3/5 obtained in the absence and presence of Nedd4-2. Data are expressed as mean  $\pm$  S.E. of 10 oocytes/group.

## RESULTS

**Nedd4-2 Regulates KCNQ2/3 and KCNQ3/5 Expression in *Xenopus* Oocytes**—Depolarization-activated  $K^+$  currents were recorded from oocytes expressing KCNQ2/3 and KCNQ3/5 heteromers. The currents exhibited similar current-voltage (I-V) relationships (see Fig. 1) and sensitivity to linopirdine (data not shown) as reported previously (4, 8, 28). Co-expression with Nedd4 did not significantly alter the current amplitude of KCNQ2/3 ( $n = 20$ ) and KCNQ3/5 ( $n = 20$ ) (Fig. 1, *A* and *B*). In contrast, Nedd4-2 caused a significant down-regulation of both KCNQ2/3- and KCNQ3/5-mediated current amplitudes without altering the I-V relationships (Fig. 1, *C* and *D*). When co-expressed with Nedd4-2, the  $K^+$  current amplitude mediated by the KCNQ2/3 heteromer was reduced to

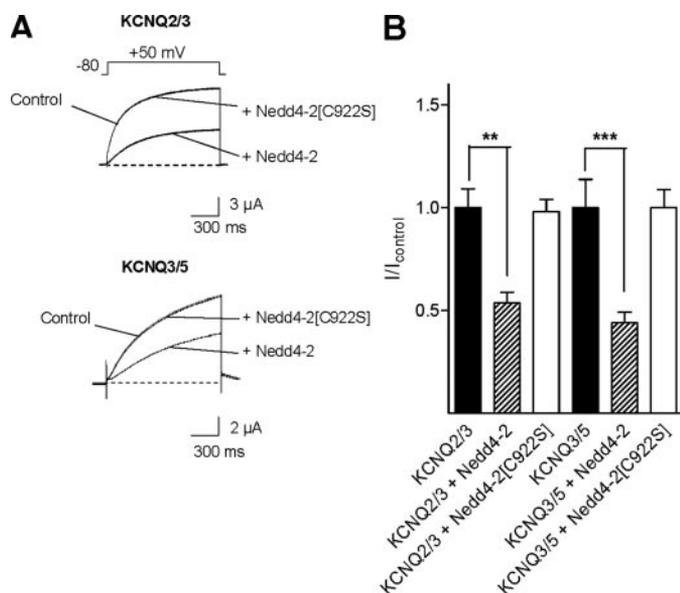
$53.6 \pm 5.2\%$  ( $n = 70$ ;  $p < 0.01$ ) of control (KCNQ2/3 expressed alone) (Fig. 1, *A* and *C*). Similar effects were observed for the  $K^+$  current mediated by the KCNQ3/5 channels, which was reduced to  $43.9 \pm 5.1\%$  ( $n = 28$ ;  $p < 0.001$ ) of control (Fig. 1, *B* and *D*).

To investigate whether Nedd4-2 regulated the homomeric KCNQ2 and KCNQ5 channels, these subunits were then co-expressed with Nedd4-2 in the absence of KCNQ3. As reported previously (4), the amplitude of  $K^+$  currents mediated by KCNQ2 and KCNQ5 alone were 5–10-fold smaller than the currents mediated by the heteromeric channels, whereas currents mediated by KCNQ3 were almost undetectable (data not shown). Nedd4-2 had no effect on the current amplitudes of KCNQ2 ( $n = 20$ ) and KCNQ5 ( $n = 10$ ) expressed alone (data not shown), suggesting that the presence of the heteromeric channel is necessary for the Nedd4-2-mediated down-regulation to occur.

To demonstrate the functional specificity of the interaction between Nedd4-2 and KCNQ2/3 and KCNQ3/5, a point mutation of cysteine $\rightarrow$ serine in the HECT domain of Nedd4-2 was tested. In contrast to Nedd4-2, the ligase-deficient Nedd4-2[C922S] had no effect on the  $K^+$  currents mediated by KCNQ2/3 ( $n = 38$ ) and KCNQ3/5 ( $n = 30$ ) (see Fig. 2), suggesting that the catalytic activity of Nedd4-2 was required for the inhibitory function of this enzyme on KCNQ2/3- and KCNQ3/5-current amplitudes.

**Nedd4-2 Down-regulates KCNQ2/3 and KCNQ3/5 Heteromers Independently of the PPXPPY Motif in KCNQ3**—The dependence of Nedd4-2-mediated down-regulation of the KCNQ2/3 and KCNQ3/5 heteromers on the PPXPPY motif in the C-terminal region of KCNQ3 was examined by the site-directed mutagenesis of this motif in KCNQ3, in which tyrosine was substituted with an alanine (Y698A). When co-expressed with KCNQ2 and KCNQ5, the channels containing KCNQ3[Y698A] exhibited a similar I-V relationship as the wt heteromers (data not shown); however, the current amplitude mediated by the KCNQ3[Y698A] channel was significantly lower than the KCNQ3 channel (Fig. 3). Similar to the wt channels, Nedd4-2 significantly reduced the current amplitude mediated by heteromeric channels containing

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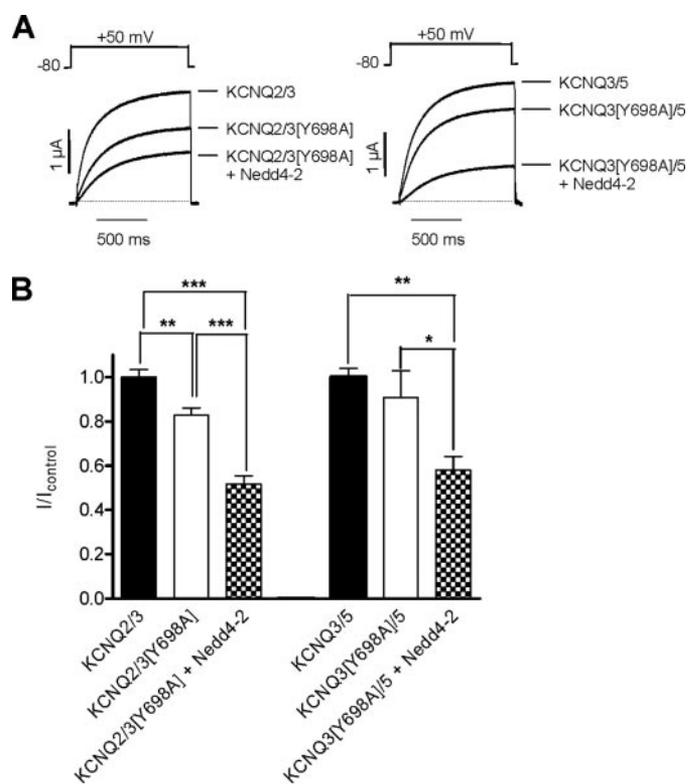


**FIGURE 2. Nedd4-2 (but not the catalytically inactive analogue Nedd4-2[C922S]) reduces KCNQ2/3- and KCNQ3/5-mediated K<sup>+</sup> current amplitude.** *A*, superimposed K<sup>+</sup> currents recorded from *Xenopus* oocytes expressing either KCNQ2/3 or KCNQ3/5 alone and in combination with Nedd4-2 and Nedd4-2[C922S]. Oocytes were held at -80 mV and depolarized to +50 mV. *B*, bar graph of normalized K<sup>+</sup> current amplitude obtained at +50 mV in oocytes expressing KCNQ2/3 alone, KCNQ2/3 in the presence of wt Nedd4-2, KCNQ2/3 expressed with Nedd4-2[C922S], KCNQ3/5 alone, KCNQ3/5 with wt Nedd4-2, and KCNQ3/5 in the presence of Nedd4-2[C922S]. *n* = 28–70 oocytes from 3–5 batches. Asterisks indicate that the current amplitudes are significantly different from control (KCNQ2/3 or KCNQ3/5 expressed alone; \*\*, *p* < 0.01; \*\*\*, *p* < 0.001).

KCNQ3[Y698A] by ~31.0 ± 8.9% (KCNQ2/3, *n* = 55, *p* < 0.001) and 32.7 ± 4.0% (KCNQ3/5, *n* = 30, *p* < 0.05) (Fig. 3*B*).

**Nedd4-2 Does Not Down-regulate KCNQ2/3 and KCNQ3/5 Heteromers Containing a Truncated KCNQ3 C Terminus**—To investigate whether the cytoplasmic C-terminal region of KCNQ3 is required for the Nedd4-2 down-regulation of KCNQ2/3 and KCNQ3/5 K<sup>+</sup> current amplitudes, a limited deletion analysis of the KCNQ3 was undertaken. The KCNQ3 subunit was truncated by deleting the last two-thirds (333 amino acid residues) of the C-terminal region at Arg-502 (Fig. 4*A*). The truncated KCNQ3 subunit (KCNQ3Δ) was co-expressed with either KCNQ2 or KCNQ5 in *Xenopus* oocytes, and K<sup>+</sup> current amplitudes were recorded in the absence and presence of Nedd4-2. The steady-state K<sup>+</sup> current amplitude obtained for both truncated KCNQ2/3 and KCNQ3/5 in the absence and presence of Nedd4-2 was not significantly different at all voltages (*n* = 4–17 oocytes) (Fig. 4, *B* and *C*). In control experiments, using the same batch of oocytes, co-expression of either KCNQ2/3 or KCNQ3/5 and Nedd4-2 resulted in a significant reduction in K<sup>+</sup> current amplitude as shown previously in Fig. 1. The failure of Nedd4-2 to reduce K<sup>+</sup> current amplitude when the C-terminal region of KCNQ3 was truncated demonstrates that the intracellular C-terminal region of KCNQ3 is involved in Nedd4-2 regulation of KCNQ2/3 and KCNQ3/5 channel expression at the plasma membrane.

**Nedd4-2 Interacts with KCNQ2 and KCNQ3 but Not via the PPXPPY Motif**—To determine whether Nedd4-2 bound directly to the C-terminal region of KCNQ3, GST fusion pull-downs were performed using the C-terminal region of



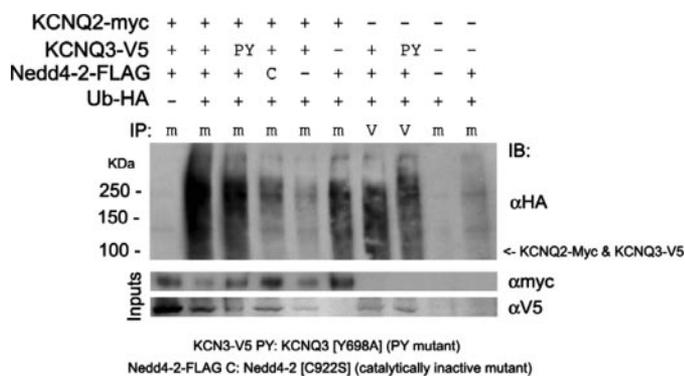
**FIGURE 3. Nedd4-2 down-regulates KCNQ2/3 and KCNQ3/5 heteromers independently of the PPXPPY motif in KCNQ3.** *A*, superimposed depolarization-activated K<sup>+</sup> currents recorded from oocytes expressing KCNQ2/3, KCNQ2/3[Y698A], KCNQ2/3[Y698A] + Nedd4-2, KCNQ3/5, KCNQ3[Y698A]/5, and KCNQ3[Y698A]/5 + Nedd4-2. Oocytes were held at -80 mV and depolarized to +50 mV. *B*, bar graph of relative K<sup>+</sup> current amplitude obtained at +50 mV from oocytes expressing KCNQ2/3 and KCNQ3/5 channels containing KCNQ3[Y698A] in the absence and presence of Nedd4-2. Data were normalized to the average of wt KCNQ2/3 and KCNQ3/5 and are expressed as mean ± S.E. of 30–55 oocytes from 4–5 batches. Asterisks indicate that the current amplitude was significantly different from the control; \*, *p* < 0.05; \*\*, *p* < 0.01; \*\*\*, *p* < 0.001.

KCNQ3 (GST-KCNQ3-CT) and KCNQ3[Y698A] (GST-KCNQ3[Y698A]-CT). GST-KCNQ3-CT and GST-KCNQ3[Y698A]-CT bound to glutathione-Sepharose beads were incubated with rat brain lysate. The proteins bound to the beads were subsequently eluted, immunoblotted, and probed with an antibody directed against Nedd4-2. The bands appeared as doublets, which are most likely because of Nedd4-2 splice variants (29). Nedd4-2 was detected in the presence of both GST-KCNQ3-CT and GST-KCNQ3[Y698A]-CT but not GST alone (Fig. 5*A*), suggesting that the interaction between Nedd4-2 and KCNQ3 is independent of the PY motif.

To demonstrate that KCNQ3 and Nedd4-2 did interact under normal cellular conditions, co-immunoprecipitation assays were carried out on lysates from HEK293T cells transfected with FLAG-tagged Nedd4-2 and V5-tagged KCNQ3 and KCNQ3[Y698A] alone and in the presence of Myc-tagged KCNQ2. Proteins interacting with Nedd4-2-FLAG were immunoprecipitated with an anti-FLAG antibody, immunoblotted, and simultaneously probed with an anti-V5 antibody to detect KCNQ3-V5 and an anti-Myc antibody to detect KCNQ2-Myc. Bands of the appropriate size were detected in the lanes loaded with lysate containing KCNQ2 (~89 kDa), KCNQ3 (~97 kDa), and KCNQ3[Y698A], demonstrating that



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**FIGURE 6. Nedd4-2 ubiquitinates KCNQ2 and KCNQ3.** HEK293T cells were transiently transfected with combinations of tagged KCNQ2, KCNQ3, and Nedd4-2 constructs and HA-tagged ubiquitin as indicated with +. Proteins were immunoprecipitated (IP) with antibodies directed against the Myc tag (as indicated by *m*) or the V5 tag (as indicated by *v*), transferred to a nitrocellulose membrane, and subjected to immunoblotting (IB) with anti-HA antibody to detect ubiquitinated species (as seen by smearing). Lanes 9 and 10 are negative controls, with Nedd1-Myc expressed in lane 10 as a negative control for ubiquitination, as Nedd1 is not a substrate for Nedd4-2. Inputs show expression levels of the KCNQ2 and KCNQ3 proteins in HEK293T cells.

nated species were detected with an anti-HA antibody. Polyubiquitination of a target protein typically results in high molecular weight smearing. High molecular weight species were detected in cells expressing KCNQ2, wt KCNQ3, or KCNQ3[Y698A] in combination with both Nedd4-2 and ubiquitin (Fig. 6). Ubiquitinated species were observed when the KCNQ channels were expressed as either monomers or heteromers. In the absence of Nedd4-2-FLAG or when the catalytically inactive Nedd4-2[C922S]-FLAG was included, only weak endogenous ubiquitination of KCNQ2-Myc and KCNQ3-V5 was observed. Thus, the binding of Nedd4-2 to the C terminus of KCNQ results in ubiquitination of the channel.

## DISCUSSION

To date, little is known regarding trafficking and turnover of the  $K^+$  channels that mediate the neuronal M-current. In the present study, we have identified ubiquitination by the ubiquitin-protein ligase Nedd4-2 as a potential regulatory mechanism for the KCNQ2/3 and KCNQ3/5 heteromultimers that constitute the molecular correlates of this current. We found that Nedd4-2 (but not the closely related ubiquitin ligase Nedd4) caused a pronounced down-regulation of the current amplitudes mediated by the heteromeric channels expressed in *Xenopus* oocytes. These data suggest that Nedd4-2 (rather than Nedd4) may be the physiological regulator as described for other ion channels. We demonstrated that Nedd4-2 interacts not only biochemically but also functionally with the KCNQ3-subunit.

The fact that Nedd4-2 can bind and ubiquitinate individual KCNQ2 and KCNQ3 monomers, whereas the down-regulation of currents was observed only with the heteromeric KCNQ2/3 channels, highlights the complex nature of the interactions between the individual subunits. It is clear from the current study and previous reports (4, 8, 28) that heteromeric KCNQ channels display higher cell surface expression levels than monomeric channels. It appears that heteromerization increases the trafficking efficiency of the channels to the membrane, and this regulation has been proposed to involve the

exposure of a previously hidden trafficking motif or the unmasking of an endoplasmic reticulum retention signal (30–32). It is therefore possible that the interaction between the C-terminal regions of KCNQ2 and KCNQ3 is also required for optimum internalization of the complex, perhaps by facilitating the binding of an accessory protein or endogenous KCNE-encoded auxiliary  $K^+$  channel subunit in *Xenopus* oocytes (33). Nedd4-2 failed to reduce  $K^+$  current amplitude when the intracellular C-terminal region of KCNQ3 was truncated, demonstrating that the interaction between Nedd4-2 and the C terminus of KCNQ3 is required for the regulation of KCNQ2/3 and KCNQ3/5 channel density in the plasma membrane.

In contrast to our findings with CIC-5 (23), the interaction between Nedd4-2 and KCNQ2/3 was not mediated via the C-terminal PPXPPY motif. This observation is consistent with previous studies demonstrating that Nedd4-2 can regulate proteins that do not possess a PY motif, including the glutamate transporter EAAT2 (18), the voltage-gated  $K^+$  channel  $K_v1.3$  (16), and the dopamine transporter (19). One possibility is that Nedd4-2 may bind to a different, yet unidentified, intracellular motif on the target protein. Previous reports have shown that sequences that are similar but not identical to the classical PY motifs can bind Nedd4-2 (14, 34), and the C-terminal regions of KCNQ2 and KCNQ3 contain several such sequences in which a proline and a tyrosine residue are localized in close proximity. In addition, accessory proteins can mediate the interaction of Nedd4/4-2 with target proteins, and a number of adapter proteins containing PY motifs that link ubiquitin ligases of the Nedd4 family to their target proteins have been identified (reviewed in Ref. 35). For example, mGrb10 $\alpha$  mediates the interaction between Nedd4 and the insulin growth factor 1 receptor (36), and Bsd2p connects the Nedd4-2 *Saccharomyces cerevisiae* homologue Rsp5p to a metal ion transporter (37). One such candidate is the chaperone KChAP, which has been shown to interact with  $K_v$  channels (38). KChAP contains a classical PY motif and may therefore mediate an interaction with Nedd4-2. It is also possible that Nedd4-2 regulates another E3 ubiquitin ligase that may act on KCNQ2/3, as has been suggested for dopamine transporter (19). In the case of dopamine transporter, ubiquitination by Nedd4-2 appears to be dependent on protein kinase C activity, and it has been suggested that phosphorylation of the dopamine transporter by protein kinase C may allow access of binding or ubiquitination sites for Nedd4-2 or a Nedd4-2-regulated ubiquitin ligase or may alternatively directly increase the activity of Nedd4-2 (19). Thus, the mechanisms by which Nedd4/Nedd4-2 can regulate substrate proteins are highly complex in terms of accessory proteins and modulation by other factors such as protein kinases.

Mutating the PY motif of KCNQ3 resulted in significantly lower  $K^+$  current amplitudes than the wt channel KCNQ2/3 heteromer. The mutated residue (Tyr-698) is localized in the proximity of the TCC2 domain in the C terminus of KCNQ3, a region known to be involved in heteromerization (32). Substitution of Tyr-698 with an alanine may result in a conformational change that could affect the assembly or stabilization of the heteromeric KCNQ2/3 complex, resulting in reduced  $K^+$  currents. Another interesting finding was that the catalytically inactive Nedd4-2 analogue (Nedd4-2[C922S]) did not appear to

interact with KCNQ2/3 as well as wt Nedd4-2. Replacement of Cys-922 with a serine may not only abolish the catalytic activity but may also affect binding to KCNQ2/3 as a result of a conformational change in Nedd4-2. This catalytically inactive Nedd4-2 mutant has been shown to have a dominant negative effect on cell surface levels of certain target proteins expressed in *Xenopus* oocytes by competing with endogenous  $\alpha$ Nedd4-2 (11, 23). We did not observe any increase in the current amplitude mediated by either KCNQ2/3 or KCNQ3/5 in the presence of Nedd4-2[C922S], suggesting that  $\alpha$ Nedd4-2 does not regulate KCNQ2/3/5 as has been shown previously for the neuronal voltage-gated Na<sup>+</sup> channel (14).

Originally thought of primarily as a regulator of ENaC, the Nedd4/Nedd4-2 ubiquitin ligases have been shown to regulate a number of ion channels, receptors and transporters in several tissues. In terms of neural cells, Nedd4-2 may be of importance in pathological conditions such as stroke when cell surface levels of neuronal ion channels change dramatically (39, 40) and in neurodegenerative diseases when proteasomal activity is altered (41, 42). Nedd4-2 has been implicated in the regulation of voltage-gated Na<sup>+</sup> channels, the internalization of the dopamine transporter, and turnover of receptors that regulate growth, differentiation, and axonal guidance (14, 43–45) and ubiquitination are potentially involved in the release of neurotransmitters (46). The potential regulation of the M-current by Nedd4-2 may be of particular importance during development as well as in the adult brain, where it is possible that Nedd4-2 plays a crucial role in the fine-tuning of M-current activity. This regulation may have profound effects on neuronal excitability, because subtle reductions (<25%) in M-current activity can alter excitability to the point of causing seizures (47). Genetic variants in human Nedd4-2 have recently been identified in patients with photosensitive epilepsy (48). Abnormal or decreased activity of the M-current is directly linked to certain disease states (not only epilepsy but also Alzheimer disease (49) and chronic pain states (10, 27)), and dysfunctional regulation of KCNQ channels by Nedd4-2 could potentially contribute to such diseases. In summary, this study describes a new paradigm for the regulation of KCNQ2/3/5 channels by the ubiquitin ligase Nedd4-2. This finding provides a novel mechanism for the modulation of the M-current with a potential role in the regulation of neuronal excitability.

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**Regulation of the Voltage-gated K<sup>+</sup> Channels KCNQ2/3 and KCNQ3/5 by Ubiquitination: NOVEL ROLE FOR Nedd4-2**

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