Peripheral neuropathic pain is a disabling condition resulting from nerve injury. It is characterized by the dysregulation of voltage-gated sodium channels (Navs) expressed in dorsal root ganglion (DRG) sensory neurons. The mechanisms underlying the altered expression of Navs remain unknown. This study investigated the role of the E3 ubiquitin ligase NEDD4-2, which is known to ubiquitylate Navs, in the pathogenesis of neuropathic pain in mice. The spared nerve injury (SNI) model of traumatic nerve injury–induced neuropathic pain was used, and an Na\textsubscript{1.7}-specific inhibitor, ProTxII, allowed the isolation of Nav1.7-mediated currents. SNI decreased NEDD4-2 expression in DRG cells and increased the amplitude of Na\textsubscript{1.7} and Na\textsubscript{1.8} currents. The redistribution of Na\textsubscript{1.7} channels toward peripheral axons was also observed. Similar changes were observed in the nociceptive DRG neurons of Nedd4L knockout mice (SNS-Nedd4L\textsuperscript{−/−}). SNS-Nedd4L\textsuperscript{−/−} mice exhibited thermal hyperalgesia and an enhanced second pain phase after formalin injection. Restoration of NEDD4-2 expression in DRG neurons using recombinant adenoassociated virus (rAAV2/6) not only reduced Nav1.7 and Na\textsubscript{1.8} current amplitudes, but also alleviated SNI-induced mechanical allodynia. These findings demonstrate that NEDD4-2 is a potent posttranslational regulator of Navs and that downregulation of NEDD4-2 leads to the hyperexcitability of DRG neurons and contributes to the genesis of pathological pain.

Authorship note: Hugues Abriel and Isabelle Decosterd contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2013;123(7):3002–3013. doi:10.1172/JCI68996.
NEDD4-2 was recently shown to be decreased in rat DRG in the spared nerve injury (SNI) model of traumatic nerve injury–induced neuropathic pain (25). The present study postulated that reduced levels of NEDD4-2 jeopardize the correct addressing or anchoring of Navs in DRG nociceptive neurons. NEDD4-2 expression was controlled in cellular expression systems and in mice with DRG-specific gene deletions or rAAV-mediated gene transfers. This enabled the selective investigation of the effect of NEDD4-2 on Nav1.7 and Nav1.8 currents, as well its impacts on pain sensitivity. The results provide what we believe to be the first in vivo mechanistic evidence that NEDD4-2 enables the fine-tuning of neuronal excitability in DRG cells. Furthermore, these results may demonstrate that the pathological reduction of NEDD4-2 underlies traumatic nerve injury–induced neuropathic pain.

Results
Peripheral nerve injury reduces NEDD4-2 expression in DRG. The protein and mRNA levels of Nedd4L were measured to explore whether Nedd4L is regulated after nerve injury in mice and whether it contributes to phenotypic changes in DRG neurons. A substantial decrease of NEDD4-2 expression was observed by immunofluorescence in lumbar L4/L5 DRG 7 days after SNI (Figure 1, A and B). This decrease was further quantified using Western blot analysis. SNI decreased NEDD4-2 protein levels by greater than 60% in DRG, an effect that lasted for at least 6 weeks (Figure 1C). Both SNI and spinal nerve ligation (SNL) reduced Nedd4L transcript levels (Figure 1D). Nedd4L mRNA was abundantly expressed in lumbar L4/5 DRG and was the only member of the Nedd4/Nedd4-like E3 subfamily to be downregulated after SNI (Figure 1E).

Figure 1
Peripheral nerve injury reduces NEDD4-2 expression in DRG. (A and B) Immunofluorescence of NEDD4-2 in coronal sections of L4 DRG from sham-operated and SNI mice. Scale bars: 30 μm. (C) Representative Western blot analysis showing the decrease in NEDD4-2 at days 7, 21, and 42 after SNI in L4/5 DRG and its associated quantification. Data are expressed as the means ± SEM; n = 4 samples for each time point per group. ***P < 0.001 by 1-way ANOVA with Bonferroni’s post-hoc test. GAPDH was used as a loading control. Lanes were run on the same gel but were noncontiguous. (D) Effect of SNI and SNL on Nedd4-1 and Nedd4L transcripts in L4/5 DRG 7 days after SNI or SNL (injury of L5 spinal nerve). Bar graph showing transcriptional levels of Nedd4-1 and Nedd4L normalized to GAPDH in SNI and SNL groups over the control group (sham for SNI and L4 DRG for SNL). Data represent the mean ± SEM; n = 4 samples per group. Isolated L4/5, L5, or L4 DRG from 2 mice were pooled for each sample and run in triplicate. *P = 0.011, **P = 0.004, Student’s t test. (E) Constitutive transcript levels of Nedd4/Nedd4-like E3 subfamily members in L4/5 DRG 7 days after sham and SNI surgery. Transcript levels were normalized using HPRT as a reference gene and further normalized to Nedd4L levels in sham-operated mice. Data are expressed as the means ± SEM; n = 3–4 samples per group, which were run in triplicate. **P = 0.005, Student’s t test. We detected no amplification of NedL1 in the DRG samples.
suggesting that NEDD4-2 mainly reduces the number of channels at the cell surface. Cell-surface proteins were then biotinylated and precipitated. Upon NEDD4-2 cotransfection, expression of the fully glycosylated form of Na_v1.7 (Supplemental Figure 1B) was decreased by approximately 50% in the plasma membrane fraction, but remained unchanged in the total lysate (Figure 2C, see also Supplemental Figure 1, C and D, for additional in vitro experiments). The interaction between Na_v1.7 and NEDD4-2 was examined by pull-down experiments using GST fused to the furthest 66 C-terminal amino acid residues of Na_v1.7, which include the PY motif (GST-Cter-Na_v1.7). Na_v1.7 GST fusion proteins interacted with endogenous and transfected NEDD4-2, whereas GST alone did not (Figure 2D). Finally, whether Na_v1.7 could be a substrate of NEDD4-2 ubiquitylating activity was tested by pulling down ubiquitylated proteins using GST fused to the ubiquitin-binding proteasomal subunit S5A (GST-S5A). Overexpression of NEDD4-2 substantially increased GST-S5A–bound Na_v1.7 (Figure 2E). Taken together, these results support a model in which NEDD4-2 interacts with and ubiquitylates Na_v1.7 and thus controls the level of the functional channel at the cellular membrane in mammalian cells. Additional in vitro experiments demonstrating the importance of the PY motif in the NEDD4-2 downregulatory effect on Nav1.7 are presented in Supplemental Figure 2, A–F and in the Supplemental Results.

SNI changes the expression of Na_v.s. In freshly dissociated L4/5 mouse DRG neurons, the different Na_v components were functionally dissected out 1 week after SNI by performing whole-cell patch-clamp recordings. From total I_Na (Navtotal), the specific Nav1.7 channel blocker ProTxII (26) and TTX were used to isolate the following 3 currents: the Na_v1.7-mediated current (ProTxII-sensitive current, referred to as Nav1.7 for simplicity), the remain-
ing TTX-sensitive currents (Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, and Na\textsubscript{v}1.6 currents collectively referred to as Nav\textsubscript{r}TTXs), and the TTX-resistant currents (referred to as Na\textsubscript{v}1.8, since Na\textsubscript{v}1.9 was inactivated by an ad-hoc electrophysiological protocol; see Methods) (Figure 3A). Recorded cells were small neurons (<30 pF), considered to be nociceptive neurons (27). Despite the fact that the distinction between intact or severed neurons was not made, a significant increase in the Nav\textsubscript{total} (\(P = 0.013\)) and Na\textsubscript{v}rTTXs (\(P = 0.021\)) current densities after SNI were measured (ipsilateral compared with the contralateral side, Supplemental Figure 3A). Since the expression of Na\textsubscript{v}s in DRG is heterogeneous, the analysis was refined by segregating cells into fast and slow neurons, as previously reported (27). A neuron was characterized as slow when the \(I_{Na}\) density ratio of the Nav\textsubscript{1.8}/Nav\textsubscript{total} was greater than 0.5, with Nav\textsubscript{1.8} displaying slower inactivation kinetics. Conversely, when this ratio was less than 0.5, the neuron was defined as fast (27). This selection revealed that SNI significantly increased Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8 current densities in the slow subpopulation only (Figure 3B). The fast subpopulation showed a small but significant increase in Nav\textsubscript{r}TTXs alone (Figure 3C; \(P = 0.014\)).

SNI had only a minor impact on the biophysical properties (voltage dependence of steady-state activation and inactivation) of some of the Na\textsubscript{v} components (Supplemental Table 2). In line with previous studies (28, 29), nerve injury induced an acceleration of the recovery from inactivation (repriming) for every component of \(I_{Na}\) of the fast subpopulation (Supplemental Table 2).

Western blots of pooled L4/5 DRG revealed no detectable modification of the expression levels of Nav\textsubscript{total}, nor that of Nav\textsubscript{1.7} (\(P = 0.039\)) or Nav\textsubscript{1.8} (\(P = 0.024\)) 1 week after SNI (Figure 3D). However, Na\textsubscript{v}1.7 and Nav\textsubscript{total} levels were significantly increased in the sciatic nerve. Nav\textsubscript{1.8} was undetectable in the nerves of sham-operated animals. The signal intensity was not significantly modified after SNI, but a distinct band at the expected molecular weight (230–240 kDa) was visible in all 4 SNI samples (see Supplemental Figure 3B).
Figure 4
SNS-Nedd4L−/− mice show increased Na1.7 and Na1.8 currents in DRG neurons and increased expression of Na1.7 along the sciatic nerve. (A and B) Immunofluorescence of NEDD4-2 in coronal sections of L4 DRG from Nedd4LLox/lox and SNS-Nedd4L−/− mice. Scale bars: 30 μm. (C and D) Immunofluorescence and corresponding bright-field images of NEDD4-2 in DRG neurons from Nedd4LLox/lox and SNS-Nedd4L−/− mice after whole-cell patch-clamp recordings (36 hours after dissociation). Scale bars: 30 μm. (E) Western blot and quantification showing NEDD4-2 decrease in the DRG (**P = 0.003) of SNS-Nedd4L−/− mice compared with control Nedd4LLox/lox mice. (F and G) Scatter dot plots representing Na_{total}, Na_{1.7}, Na_{1.8}, and Na_{rTTX} currents densities in L4/5 DRG neurons from SNS-Nedd4L−/− and Nedd4LLox/lox mice. Slow (F, in cyan) and fast (G, in magenta) neurons are shown. Mann-Whitney U test. See Supplemental Figure 4A for total population and Supplemental Table 3 for values and biophysical properties. (H) Left panel: Western blot analysis and quantification of Na_{α} subunits in the DRG of SNS-Nedd4L−/− and Nedd4LLox/lox mice. No significant modifications in Na_{total} (P = 0.054) or Na_{1.7} (P = 0.646) were observed, whereas the Na_{1.8} signal was increased in the SNS-Nedd4L−/− mice (**P = 0.020). Right panel: same as above, but for sciatic nerves. Na_{1.7} was significantly increased (**P = 0.022), whereas the increase in Na_{total} was not significant (P = 0.089). Data are expressed as the means ± SEM; n = 4 samples for each group. Student’s t test. Tubulin was used as a loading control in E and H.
Nav expression in SNS-Nedd4L−/− knockout mice. To investigate the contribution of NEDD4-2 to the expression of Navs in DRG in vivo and its impact on pain, a DRG neuron-specific Nedd4L-deficient mouse line was generated (SNS-Nedd4L−/−; see Supplemental Methods). Mice carrying a homozygous Nedd4L flox allele (Nedd4Lfl/fl) (30) were crossed with mice heterozygously expressing Cre recombinase under the control of the Na1.8 promoter (referred to as SNS-Cre), which is predominantly active in DRG nociceptive neurons (31). Cre expression in this mouse line has been extensively characterized (32) and differs from another Na1.8-Cre mouse line generated by the Wood laboratory (33). NEDD4-2 expression was greatly reduced in the DRG neurons of SNS-Nedd4L−/− mice (Figure 4, A–E). A slight signal was still detectable in the SNS-Nedd4L−/− mice, most likely due to a residual expression of NEDD4-2 in neurons not expressing Na1.8 (Figure 4E). Whole-cell patch-clamp recordings showed a 2-fold upregulation of Na1.7 (P = 0.027), and Na1.8 (P < 0.001) current densities in neurons from SNS-Nedd4L−/− mice (Supplemental Figure 4A and Supplemental Table 3) compared with neurons from Nedd4Lfl/fl control littermates. The Na1.8 current was not significantly altered. Subsequent analyses of slow and fast neuronal subpopulations revealed that, similar to the SNI condition, the changes were predominant in slow neurons (a 2-fold increase for Na1.total [P = 0.001], Na1.7 [P = 0.042], and Na1.8 [P < 0.001] current densities) (Figure 4, F and G). The biophysical properties were largely unaltered in the knockout mice (Supplemental Table 3), consistent with a major role of NEDD4-2 in the regulation of Na1.7 and Na1.8 membrane density.

We then evaluated whether the expression of Navs was modified in DRG and the sciatic nerves of SNS-Nedd4L−/− mice (Figure 4H). We observed a markedly increased Western blot signal for Na1.8 in the DRG of knockout mice, while the signal was undetectable in Nedd4Lfl/fl DRG neurons. These observations were confirmed by immunofluorescence measurements (Supplemental Figure 4B). Although the Na1.7 signal from DRG was not changed between the groups, a stronger signal was observed in the sciatic nerves of the SNS-Nedd4L−/− mice, similar to that observed after SNI. Na1.8 immunoreactivity was not detected in the sciatic nerves of the knockout mice or in those of the control mice, suggesting that the mechanisms underlying Na1.8 redistribution in nerves after SNI are independent of NEDD4-2 downregulation.

Pain-related responses in SNS-Nedd4L−/− knockout mice. We then investigated whether peripheral deficiency of NEDD4-2 could modify pain behavior. While response latencies to radiant heat (tail-flick test) (Figure 5B) and acute mechanical nociception (tail pressure test) (Figure 5C) were unchanged, acute thermal hypersensitivity in SNS-Nedd4L−/− mice was observed with the hot-plate test at 52°C and 55°C (Figure 5A). We also performed the hot-
plate test at 52°C after SNI (Figure 5E). **Nedd4L<sup>fl/fl</sup>** mice did not develop SNI-induced hyperalgesia. The observed thermal hyperalgesia in the **SNS-Nedd4L<sup>-/-</sup>** mice under basal conditions was not further enhanced after SNI. The response of the hot-plate test depends on the strength applied by the paw to the plate, which may be impeded after SNI surgery due to the transection of motor neurons. To overcome this problem, we performed the plantar test, an alternative thermal sensitivity test independent of strength, at the end of the SNI time course (i.e., 35 days after SNI). As illustrated in Figure 5F, the noninjured paws of **SNS-Nedd4L<sup>-/-</sup>** mice demonstrated more thermal hypersensitivity than the noninjured paws of **Nedd4L<sup>fl/fl</sup>** mice. SNI induced thermal hypersensitivity in the injured paws of **Nedd4L<sup>fl/fl</sup>** mice, but did not further enhance the hypersensitivity in the injured paws of **SNS-Nedd4L<sup>-/-</sup>** mice. Basal mechanical innocuous sensitivity was not altered in **Nedd4L<sup>fl/fl</sup>** mice (see baseline [BL] in the von Frey filaments test in Figure 5D). After SNI, the decrease in the withdrawal threshold related to the development of mechanical allodynia–like behavior was indistinguishable between groups. Intraplantar injection of formalin in **SNS-Nedd4L<sup>-/-</sup>** mice led to an increased and earlier maximal

**Figure 6**

Delivery of rAAV2/6-NEDD4-2 viral vector decreases functional currents after SNI and alleviates mechanical allodynia. (A–D) Immunofluorescence of NEDD4-2 in coronal sections of L4 ipsilateral DRG injected with rAAV2/6-NEDD4-2 or saline solution in sham- and SNI-operated mice. Scale bars: 30 μm. (E and F) Scatter dot plots representing Na<sub>total</sub>, Na<sub>1.7</sub>, Na<sub>1.8</sub>, and Na<sub>1.rTTXs</sub> current densities 1 week after SNI in noninfected DRG neurons (NINF), rAAV2/6-NEDD4-2–infected cells (INFNEDD4), and in the control group infected with the rAAV2/6-NEDD4-2CS vector (INFNEDD4-2CS). Slow (E, in cyan) and fast (F, in magenta) neurons are shown. Nonparametric 1-way ANOVA (Kruskal-Wallis test) with Dunn’s post-hoc test. See Supplemental Figure 5F for total population, Supplemental Table 5 for biophysical properties and values, and Supplemental Figure 5, A–E. (G) Basal thermal sensitivity showed no difference at 49°C (P = 0.987), 52°C (P = 0.186), or 55°C (P = 0.673) in the hot-plate test between the 2 groups. Student’s t-test. (H) An increase in tail-flick latency (P = 0.018 at intensity 7) for high-intensity stimulation in the rAAV2/6-NEDD4-2 group was observed. Mann-Whitney U test. (I) Tail pressure sensitivity was increased in mice infected with rAAV2/6-NEDD4-2. *P = 0.006, Student’s t-test. (J) Basal responses to innocuous mechanical stimulation were not different between the 2 strands, but the development of mechanical allodynia was significantly diminished in rAAV2/6-NEDD4-2–infected mice. ***P < 0.001 at day 7 and **P < 0.01 at day 14; 2-way ANOVA on log values with post-hoc Bonferroni’s tests. Data are expressed as the mean ± SEM; n = 12–15 for rAAV2/6-stuffer and rAAV2/6-NEDD4-2.
response (peak at 30.4 ± 1.4 minutes in SNS-Nedd4L−/− mice compared with 36.3 ± 1.3 minutes in Nedd4L−/+ mice; P = 0.039) during the early second phase as compared with the Nedd4L−/+ mice, suggesting a central consequence of increased peripheral activity due to Nedd4L deletion (Figure 5G). Acute nociception during phase 1 of the formalin test was similar between the groups.

Functional effects of in vivo exogenous NEDD4-2 overexpression. To test whether the rescue of NEDD4-2 reduction in DRG may functionally modify Na+, expression and influence the course of SNI-induced hypersensitivity, we generated a recombinant serotonin 6 adenoeassociated viral vector expressing NEDD4-2 (rAAV2/6-NEDD4-2, Supplemental Figure 5A). Two control vectors were designed: noncoding (stuffer) vectors and those expressing the catalytically inactive form of NEDD4-2 (NEDD4-2CS). The infection efficiency of the viral vector was recently demonstrated for small DRG neurons (presumably nociceptors) after intrathecal delivery, and the transduction efficiency was shown to be entirely preserved after peripheral nerve injury (34). Immunofluorescence experiments showed that rAAV2/6-NEDD4-2 increased NEDD4-2 expression in L4/5 DRG under naive conditions and after SNI (Figure 6, A–D and Supplemental Figure 5B). INa measurements were taken of small DRG neurons after Nedd4L gene delivery, followed by single-cell PCR to identify infected (INF) and noninfected neurons (NINF). In naive animals, NEDD4-2 overexpression altered neither the INa density, nor the biophysical properties of rAAV2/6-NEDD4-2-infected DRG neurons when compared with cells infected with the control vector (INFNEDD4-2CS or the NINF neurons [Supplemental Figure 5, C–E, and Supplemental Table 4]). In the SNI condition, exogenous expression of NEDD4-2 substantially reduced the INa,q densities for Na+ total (by 49%), Na+1.7 (by 53%), and Na+1.8 (by 58%) of infected neurons (INFNEDD4-2). This was observed mainly in the fast subpopulation of DRG neurons as compared with the controls (INFNEDD4-2CS and NINF; Figure 6F and Supplemental Table 5), accounting for the tendency observed in the total population (Supplemental Figure 5F). The virus can potentially transduce all types of DRG cells, which may explain why effects were observed only in the fast population and not in the slow population. Before determining whether restoring NEDD4-2 expression with this viral vector would impact NAMediated hypersensitivity, we first tested the basal sensitivity. The response to innocuous mechanical stimuli remained unchanged (see baseline in the von Frey filaments test; Figure 6A). Responses to the hot-plate test (Figure 6G) and the low intensity of the tailflick tests were not different between rAAV2/6-NEDD4-2 or control vector (rAAV2/6-stuffer) mice (Figure 6H). However, mice infected with rAAV2/6-NEDD4-2 showed mild, but significant, hyposensitivity to the higher stimulus of the tail-flick test, whereas they showed mild, but significant, hypersensitivity to mechanical nociception (tail pressure test; Figure 6I). The development of mechanical allodynia following SNI was repressed in rAAV2/6-NEDD4-2–infected mice as compared with mice infected with the noncoding vector (Figure 6J), highlighting the behavioral consequences of the decrease in INa,q current.

Discussion

The present study provides what we believe to be the first in vivo evidence that the ubiquitin ligase NEDD4-2 exerts a strong influence on the neuronal excitation of the sensory system, and that dysregulation of this regulatory mechanism contributes to pain hypersensitivity. This is mainly due to a pathological redistribution of Na+1.7 and Na+1.8 in DRG cells.

Peripheral neuropathic pain develops across different disease states as a result of different mechanisms and depends on multiple etiological factors. In this study, we used the SNI model, a common traumatic nerve injury–induced neuropathic pain animal model (35). Although this may not mimic the mechanisms of other neuropathic pain syndromes, its fast onset and prolonged maintenance of thermal and mechanical hypersensitivity renders it very valuable.

Using this model of neuropathic pain in mice, NEDD4-2 expression was found to be substantially decreased in DRG neurons, with a concomitant increase in Na+1.7 and Na+1.8 current densities. The results also demonstrated that knocking out NEDD4-2 expression in nociceptive neurons increased Na+1.7 and Na+1.8 levels and enhanced basal pain sensitivity. Conversely, the overexpression of NEDD4-2 using rAAV2/6 vectors led to a reduction in Na+1.7 and Na+1.8 current densities and a decrease in neuropathic pain–like allodynia in the SNI model.

The different components of INa were identified in mouse DRG neurons by the use of specific electrophysiological protocols and toxins. Na+1.7-mediated currents were isolated with the perfusion of ProTxII (26). SNI increased Na+1.7 and Na+1.8 currents, particularly in the slow neuron subpopulation. Because the biophysical properties of the different INa components were not modified in the slow subpopulation, it is unlikely that the increased current density encountered after SNI was due to a modification of single-channel properties. The results suggest that an increased number of functional channels at the cell membrane may be responsible, a mechanism to which NEDD4-2 downregulation may contribute. This hypothesis is supported by the results obtained with the SNS-Nedd4L−/− mice, in which an increase in Na+1.7/1.8 current densities was also observed in slow neurons and is unlikely due to the modification of single-channel properties. The specific roles of fast/slow neuronal subpopulations are not yet understood, but the present observations suggest a role for the slow population in modulating thermal sensation.

The protein levels in DRG result from the large intracellular pool of Na+ and the small Na+ fraction at the plasma membrane (36). As a consequence, modifications of Na+ expression that would be restricted to the plasma membrane may be below the sensitivity limits of the assay, thus accounting for the lack of observed modification of Na+ expression in DRG. Interestingly, both SNI and SNS-Nedd4L−/− mice showed an accumulation of Na+1.7 along the sciatic nerve, suggesting NEDD4-2 involvement in channel axonal trafficking. The generation of SNS-Nedd4L−/− mice enabled us to investigate the functional contribution of NEDD4-2 in pain pathways. These mice exhibit an abnormal pain phenotype with increased noxious heat sensitivity, as revealed in both the hot-plate and plantar tests under basal conditions. Interestingly, the increase in thermal hypersensitivity seen with the plantar test in the SNS-Nedd4L−/− mice reached similar levels to those seen after SNI in the injured paws of control littermates. This finding suggests that genetic disruption of NEDD4-2 leads to thermal pain hypersensitivity similar to that observed when NEDD4-2 is pathologically decreased after SNI. Because SNS-Nedd4L−/− mice did not develop mechanical allodynia under basal conditions, the decrease in NEDD4-2 is probably not sufficient to render these mice mechanically hypersensitive. Neither thermal nor mechanical hypersensitivity was enhanced in SNS-Nedd4L−/−
mice after SNI, suggesting that a maximum effect of SNI-induced hypersensitivity was reached. This is consistent with the fact that NEDD4-2 cannot be further decreased, or only to a minor extent, in the DRG of SNS-Nedd4L−/− mice. Finally, the responses in the early second phase of the formalin test were increased, and the kinetics were accelerated in SNS-Nedd4L−/− mice when compared with their controls. This result suggests that the peripheral deletion of NEDD4-2 enhances noxious/nociceptive inputs in the dorsal horn and that it may be sufficient to impact the global mechanisms of central sensitization (37).

The pain behavior of the SNS-Cre mouse line does not differ from that of wild-type littermates (38), suggesting that the observed hypersensitive phenotype is due to the deletion of NEDD4-2 and not to the expression of Cre recombinase in Na1.8-positive nociceptors. The possibility that these differences may also involve non-nociceptive neurons that are potentially subject to Cre recombination cannot be ruled out, as Na1.8 expression was recently shown to extend to larger DRG neurons (39).

Major modifications of Na1.8 and Na1.7 density and distribution were observed in this study. Despite their well-established roles in nociception, the mechanisms by which Na1.7 and Na1.8 isoforms specifically contribute to neuropathic pain are still under debate. On the one hand, knocking down Na1.8 prevents neuropathic pain in mice (40), Na1.7 accumulates in human painful dental pulp (15), and gain-of-function mutations of Na1.7 and Na1.8 are linked to exaggerated pain in humans (12, 13). A recent simulation study suggested that Na1.7 and Na1.8 may act synergistically to increase the amplitude of subthreshold oscillations and increase the frequency of repetitive firing in the periphery (41). Increasing their expression in slow neurons might promote hyperexcitability. However, in sensory neuron-specific knockouts of Na1.7, Na1.8 or double-knockout mice, neuropathic pain—like behavior still develops after nerve injury (18–20). It must be noted that compensatory effects in the expression of the different Na isoforms in genetically modified animals during development cannot be excluded. The accumulation of Na1.7 and Na1.8 observed along the sciatic nerve after SNI has already been reported for Na1.8 in an experimental neuropathic pain model and was reported to contribute to neuropathic pain (14).

In the present study, counteracting the SNI-mediated decrease in NEDD4-2 using gene transfer further supports the functional importance of this ubiquitin ligase in traumatic nerve injury–induced neuropathic pain. Gene transfer has already been successfully used in mice to alleviate pain (42), and clinical trials with vectors engineered to express the proenkephalin gene for treating cancer pain are underway (43, 44). In this study, rAAV-mediated overexpression of NEDD4-2 led to a decrease in Na1.7 and Na1.8 current densities in fast DRG neurons, which was concomitant with the prevention of the full development of mechanical allodynia. It may be that rAAV overexpression of NEDD4-2 after nerve injury prevents an excess of abnormal peripheral input and reduces activity-dependent central sensitization. It is unlikely that the effects on mechanical allodynia are due to peripheral changes in the activation threshold of low-threshold mechanical afferent fibers, since basal mechanical sensitivity was minimally or not at all affected by rAAV-NEDD4-2 (nor was it after NEDD4-2 knockout). The unexpected mechanical hypoalgesic effect of rAAV2/6-NEDD4-2 might be due to the ability of ubiquitin ligase to regulate other ion channels, such as voltage-gated potassium or chloride channels (45), which could inversely affect cellular excitability depending on the targeted neuronal subpopulation. Despite its effect on the Ina of fast DRG neurons, other effects of the viral vector rAAV2/6 on cells or fibers in the peripheral nerve cannot be ruled out.

NEDD4-2 was identified as a central in vivo posttranslational regulator of Na1.7 and Na1.8, whose altered function may contribute to the development of neuropathic pain. Given that the abnormal functioning of sodium channels is a key event in the etiology of neuropathic pain, these results support a new paradigm in the treatment of this pathology. Ubiquitination-dependent mechanisms have already been implicated in neuropathic pain in a study reporting that the intrathecal delivery of proteasome inhibitors attenuated hyperalgesia in rats (46). Another posttranslational modification of sodium channels induced by the accumulation of the glycolytic metabolite methylglyoxal was recently found to play an important role in diabetic neuropathy (47). Posttranslational modifications of Na1.8 accounted for small, but significant, changes in the biophysical properties of the channel and were responsible for increased excitability of primary sensory neurons and sensitivity in diabetic mice. These results, together with the findings of this study, strongly support the need to look for agents that can modulate Na1 function and that can act as alternatives to the Na1 blockers currently used to treat neuropathic pain.

The factors that lie upstream of the observed NEDD4-2 decrease remain to be identified. Similar to many downregulated genes after SNI, axonal injury and the deprivation of trophic factors from the target tissue likely influence the transcriptional mechanisms involved in the NEDD4-2 decrease (48).

These results point to NEDD4-2 as a central regulator of nociception and demonstrate that NEDD4-2 dysfunction leads to pathological pain. The enhancement of NEDD4-2 activity may provide a novel mechanistic alternative to sodium channel blockers for the treatment of neuropathic pain. NEDD4-2 may even be involved in other neurological diseases linked to altered Na1 channel activity, such as epilepsy and migraine headaches.

Methods

DNA constructs
Human Nedd4L (KIAA0439) cDNA lacking a C2 lipid–binding domain cloned into pcDNA3.1 was a gift from T. Nagase (Kazusa DNA Research Institute, Kisarazu, Japan). Na1.7 cDNA cloned into pCIN5h was provided by S. Tate (Convergence Pharmaceuticals). The QuickChange mutagenesis kit (Stratagene) was used to generate Na1.7 and NEDD4-2 mutants. Na1.7 C-terminal PY mutants were generated as follows: Pro1944 was mutated into Ala to generate the PA mutant, Tyr1947 into Ala to generate the YA mutant, and Val1950 into Ala to generate the VA mutant. The NEDD4-2CS mutant was generated by mutating Cys401 into a Ser. shRNA against NEDD4-2 cloned into pGPZ lentiviral vector was obtained from Open Biosystems. For pull-down experiments, the cDNAs encoding the 66 last amino acids of Na1.7, the 3 different PY mutants, and the ubiquitin-binding proteosomal subunit 55A (GST-SSA) were cloned into pGEX-4T1 (Amersham Bioscience) to generate GST fusion proteins.

Western blots, immunofluorescence
See Supplemental Methods.
Pull-down and ubiquitylation experiments

pGEX-4T1 containing GST fused to the 66 last amino acids of Na1.7 WT and PY mutants, as well as GST fused to ubiquitin-binding proteasomal subunit SSA proteins, were produced (see Supplemental Methods). Pulldown proteins were analyzed by Western blot.

Cell-surface biotinylation

HEK293 cells were incubated for 30 minutes at 4°C with biotin solution (0.5 mg/ml biotin in cold PBS; Pierce Biotechnology) and then rinsed 2 times with PBS containing 200 mM glycine followed by 2 rinsings with PBS. Cells were then solubilized for 1 hour at 4°C on a wheel, and 50 μl of streptavidin-neutravidin-secpharose beads (Invitrogen) was incubated in this fraction for binding to biotinylated proteins. Samples were analyzed by Western blotting.

Real-time RT-PCR

See Supplemental Methods.

Cell culture and transfection

See Supplemental Methods.

Neuron primary culture

See Supplemental Methods.

Mouse lines

See Supplemental Methods.

Animal surgery

In the SN1 experiments, the biceps femoris was incised, exposing the sciatic nerve. The tibial and common peroneal nerves were ligated with a silk suture (Ethicon) and transected (35).

Viral vector and intrathecal injection

See Supplemental Methods.

Behavioral pain tests

Plantar test. The plantar test was conducted by exposing the lateral plantar surface of the paw to a beam of radiant heat through a transparent surface. The heat stimulation was repeated 3 times for each paw, and the mean latency time was calculated.

Hot-plate assay. The hot-plate assay was conducted by placing the animals on the hot-plate surface at varying temperatures (49°C, 52°C, and 55°C). The latency of response (in seconds) was determined by a hind paw lick or jump. The cutoff was adjusted for each temperature to avoid tissue damage (60 seconds for 49°C, 30 seconds for 52°C, and 20 seconds for 55°C).

Tail-flick assay. The tail-flick assay was conducted using a tail-flick anal- surface. The heat stimulation was repeated 3 times for each paw, and the tar surface of the paw to a beam of radiant heat through a transparent

Activation, SSI, and RFI curves were obtained as described in the in vitro analysis. The external and internal solutions used were as previously described (ref. 23 and see Supplemental Methods). Data were recorded using a VE-2 amplifier (Alemic Instruments) or an Axon 700A amplifier and analyzed using pClamp software, version 8 (Molecular Devices), KaleidaGraph, version 4.03 (Synergy Software), and MATLAB (The MathWorks). The resistance of the borosilicate pipettes (World Precision Instruments) was 2–6 MΩ. The leakage current was subtracted using the P/4 procedure.

\[ I_{Na} \text{ densities (pA/pF)} \] were obtained by dividing the peak \( I_{Na} \) by the cell capacitance obtained from the pClamp function. Current densities were normalized to WT Na1.7 or pcDNA3.1 (empty vector control) in the stable cell line for each day of the experiment. The Na+ current for the steady-state activation (SSA) curves was evoked from a holding potential of -100 mV to test pulses of 100 ms ranging from -120 mV to +30 mV in increments of 5 mV. Steady-state inactivation (SSI) curves were measured from a holding potential of -120 mV using 500-ms prepulses to the indicated potentials followed by a test pulse to 0 mV. To quantify the voltage dependence of SSA and SSI, data from individual cells were fitted with the Boltzmann relation- ship, \( y(Vm) = 1 / (1 + \exp((Vm - V_{1/2}) / k)), \) where \( y \) is the normalized current or conductance, \( Vm \) is the membrane potential, \( V_{1/2} \) is the voltage at which half of the available channels are inactivated, and \( k \) is the slope factor. The recovery from inactivation (RIF or “repriming”) curves were obtained with a standard 2-pulse protocol consisting of a depolarizing pulse from a holding potential of -120 mV to 0 mV for 50 ms to inactivate the channels, followed by a variable duration (from 0.5 ms to 3,000 ms) step back to -120 mV to promote recovery. The availability of the channels was assessed with the first standard test pulse at 0 mV, and the normalized currents of the second pulse at 0 mV were plotted versus the recovery interval. We calculated the \( t_{1/2} \) (ms), which is the time necessary for half of the channels to recover from the first pulse, by interpolation from a linear relation between the 2 points juxtaposing half recovery \( (y_1, 0.5 \cdot y_2) \), using the relation \( x = (0.5 \cdot (y_2 - y_1) / (y_2 - y_1)) \times x_1 / (y_2 - y_1) \).

Intrathecal injections. Twelve hours after plating, we performed whole-cell recordings of small neurons \((C_m \sim 30 \mu F)\) from L4/5 DRG, thought to be nociceptors (27). We used an EPC-10 amplifier and Patchmaster software (both from HEKA Electronics) for data acquisition and analysis. The external and internal solutions used were as previously described (ref. 49 and see also Supplemental Methods). Pipettes had a resistance of less than 3 MΩ, capacity transients were cancelled, and series resistance was compensated by approximately 90%. We used data only from cells in which the access resistance remained stable throughout the duration of the experiment. The leakage current was digitally subtracted using the P/4 procedure.

\( I_{Na} \) densities (pA/pF) were obtained by dividing the peak \( I_{Na} \) by the cell capacitance obtained from the HEKA function. Once in whole-cell configura- tion, cells were held at -60 mV for 5 minutes for the following reasons: (a) to dialyze the cell with CsF solution; (b) to reach equilibrium of Na1.8 (steady-state activation is shifted to hyperpolarized potentials during the first few minutes because of CsF); and (c) to lastingly inactivate the Na1.9 current (50) in order to prevent contamination of the Na1.8 current. Cells were clamped at -80 mV for 2 more minutes before starting the recordings. Activation, SSI, and RFI curves were obtained as described in the in vitro experiments, except that each pulse was preceded by a prepulse of 3 sec- onds at -120 mV to promote recovery of every Na1.8 isoform.
Pharmacological separation of Na1.7, Na1.8, and rTTXs sodium currents. Na1total current-voltage (I-V), Na1total SSI, and Na1total RFI curves were obtained as mentioned above for the total Na1 isoform component present in DRG neurons. ProTxII (5 nM; provided by B. Priest, Merck Serono), a selective blocker of Na1.7, was then perfused, and a test pulse at 0 mM was performed until the diminution of peak amplitude reached its steady state (toxin maximal effect). A 5-nM concentration of the toxin would block over 90% of Na1.7 and less than 10% of the other isoforms (26). Na1total [ProTxII] I-V, Na1total [ProTxII] SSI, and Na1total [ProTxII] RFI were then recorded. Subtracting the total curves for Na1total I-V, Na1total SSI, and Na1total RFI from those of Na1total [ProTxII] I-V, Na1total [ProTxII] SSI, and Na1total [ProTxII] RFI allowed us to measure Na1.7 I-V, Na1.7 SSI, and Na1.7 RFI. Finally, TTX (300 mM) was added to isolate the Na1.8 I-V, Na1.8 SSI, and Na1.8 RFI curves. Subtracting the Na1.8 I-V, Na1.8 SSI, and Na1.8 RFI curves from those of Na1total I-V, Na1total SSI, and Na1total RFI allowed us to record Na1rTTXs I-V, Na1rTTXs SSI, and Na1rTTXs RFI, representing the remainder of the TTX-sensitive current. For examples of this protocol for I-V curves, see figure 3A.

Statistics

For in vitro experiments (current densities, biophysical properties, and protein quantification), data were analyzed using an unpaired, 2-tailed Student’s t test when 2 groups were compared, or 1-way ANOVA for multiple group comparisons. For ex vivo recordings (current densities and biophysical properties) and behaviorial pain tests, normality was tested with a D’Agostino-Pearson omnibus test to determine whether parametrical (Student’s t) or nonparametrical (Mann-Whitney U) tests would be used when 2 groups were compared. The same normality test was performed for multiple group comparisons to determine whether a regular 1-way ANOVA and post-hoc Bonferroni’s tests, or the nonparametric equivalent test (Kruskal-Wallis and Dunn’s post tests) would be performed. For behavioral pain time courses (von Frey filaments, formalin), 2-way ANOVA with repeated measures were performed. The statistical tests used are described in each figure legend. A P value less than 0.05 was considered significant.

Study approval

All experimental procedures were approved by the Committee on Animal Experimentation of the Canton de Vaud, Switzerland, in accordance with the Swiss Federal Laws on Animal Welfare and the guidelines of the International Association for the Study of Pain (Zimmermann, 1983). Animals were housed under a 12-hour light/12-hour dark cycle and had free access to food and water.

Acknowledgments

We thank O. Staub (Lausanne University) for providing NEDD4-2 purified antibody and the Nedd4L[+/−] mouse line; R. Kuner (Heidelberg University) for providing the SNS-Cre mouse line; S. Tate and V. Morisset (Convergence Pharmaceuticals) for providing Na1.7 DNA cloned into pCIN5H and HEK293 cells stably expressing Na1.7; and B. Priest (Merck Serono) for providing ProTxII. The monoclonal antibody N68/3 (anti-Na1.7) was developed by and/or obtained from the UC Davis/NIH Neuro-Mab Facility, supported by an NIH grant (U24NS050606) and maintained by the Department of Neurobiology, Physiology and Behavior, College of Biological Sciences, University of California, Davis. We thank P. Aebischer (EPFL) for all the work performed in his laboratory and for his scientific advice. We thank M.R. Suter (CHUV and University of Lausanne), R.R. Ji (Duke University), C.J. Woolf (F.M. Kirby Neurobiology Center, Children’s Hospital Boston and Harvard Medical School), and A. Fellely (La Tour-de-Peilz, Switzerland) for comments on the manuscript. This study was supported by grants from the Swiss National Science Foundation (31003A-124996 to I. Decosterd and 31003B-135693 to H. Abriel), the Synopsis Foundation (to I. Decosterd and H. Abriel), the European Society of Anesthesiology (to I. Decosterd), and the Lemanic Neuroscience Doctoral School PhD Fellowship (to C.J. Laedermann). The contribution of B. Gavillet to the preliminary experiments is acknowledged. We would also like to thank C. Kern (CHUV and University of Lausanne) for his support.

Received for publication January 24, 2013, and accepted in revised form April 19, 2013.

Address correspondence to: Hugues Abriel, Department of Clinical Research, University of Bern, Murtenstrasse 35, 3010 Bern, Switzerland. Phone: 41.31.6320928; Fax: 41.31.6320946; E-mail: Hugues.Abriel@d kf.unibe.ch. Or to: Isabelle Decosterd, Pain Center, University Hospital Center (CHUV) and University of Lausanne, Bugnon 46, 1011 Lausanne, Switzerland. Phone: 41.21.3142040; Fax: 41.21.214 3044; E-mail: Isabelle.Decosterd@chuv.ch.