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Neuronal expression of the ubiquitin ligase Nedd4-2 in rat dorsal root ganglia: modulation in the SNI model of neuropathic pain

THESE

préparée sous la direction du Professeur associé Isabelle Décosterd

et présentée à la Faculté de biologie et de médecine de
l'Université de Lausanne pour l'obtention du grade de

DOCTEUR EN MEDECINE

par

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Médecin diplômé de la Confédération Suisse
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Neuronal expression of the ubiquitin ligase Nedd4-2 in rat dorsal root ganglia: modulation in the SNI model of neuropathic pain

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de la Faculté de Biologie et de Médecine

Madame le Professeur Stephanie Clarke
Directrice de l'Ecole doctorale

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Service d'anesthésiologie
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Rapport de synthèse

La douleur neuropathique est une forme de douleur chronique apparaissant suite à des lésions du système nerveux somato-sensoriel. Caractérisée par une plasticité neuronale inadaptée, elle est très souvent intense, invalidante, associe des symptômes comme l'allodynie ou l'hyperalgésie et reste difficile à traiter avec les agents thérapeutiques actuels.

Le thème de mon travail de thèse se concentre sur des mécanismes moléculaires de modulation des canaux sodiques voltage-dépendants suite à une lésion du nerf périphérique.

Dans l'article présenté en annexe, j'ai focalisé mon travail sur une protéine, Nedd4-2, qui est une ligase ubiquitine. Elle a pour rôle de réguler et d'internaliser dans la cellule des protéines membranaires dont les canaux sodiques. Suite aux lésions du système nerveux périphérique, il existe une hyperexcitabilité neuronale engendrée notamment par un surplus et une dysrégulation des canaux sodiques à la membrane cellulaire. Dans l'hypothèse que l'ubiquitine ligase Nedd4-2 soit présente dans les neurones sensitifs primaires et ait un rôle dans la régulation des canaux sodiques, nous avons identifié cette protéine dans les neurones nociceptifs primaires du rat. En utilisant des techniques de Western Blot et d'immunohistochimie, j'ai trouvé que Nedd4-2 est présente dans presque 50% des neurones du ganglion spinal et ces neurones sont principalement des neurones nociceptifs. Dans un modèle expérimental de douleur neuropathique (SNI, pour spared nerve injury), Nedd4-2 se retrouve significativement diminuée dans le tissu du ganglion spinal. J'ai également investigué l'expression de 2 isoformes des canaux sodiques connues pour leur implication dans la douleur, $Na_v1.7$ et $Na_v1.8$, et ces 2 isoformes se retrouvent dans les mêmes neurones que Nedd4-2. La caractérisation détaillée est décrite dans le manuscrit:

« Neuronal expression of the ubiquitin ligase Nedd4-2 in rat dorsal root ganglia: modulation in the SNI model of neuropathic pain; *Cachemaille M, Laedermann CJ, Pertin M, Abriel H, Gosselin RD, Decosterd I.* »

Les résultats obtenus indiquent que Nedd4-2, en étant downrégulé après une lésion nerveuse, pourrait ainsi contribuer à une augmentation des canaux sodiques fonctionnels à la membrane. Ainsi Nedd4-2 pourrait être proposée comme cible thérapeutique de manière alternative aux bloqueurs de canaux sodiques.

Ce travail a permis l'initiation d'autres expériences. J'ai contribué activement à la construction de vecteurs viraux type adéno-associé recombinant (rAAV2/6) et surexprimé la protéine in vivo dans les ganglions spinaux. Cette partie de mon travail se trouve intégrée dans d'autres travaux de mon laboratoire d'accueil qui a pu démontrer les effets fonctionnels de cette approche sur les courants sodiques enregistrés par électrophysiologie et une diminution de la douleur neuropathique chez la souris.

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NEURONAL EXPRESSION OF THE UBIQUITIN LIGASE NEDD4-2 IN RAT DORSAL ROOT GANGLIA: MODULATION IN THE SPARED NERVE INJURY MODEL OF NEUROPATHIC PAIN

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Abstract—Neuronal hyperexcitability following peripheral nerve lesions may stem from altered activity of voltage-gated sodium channels (VGSCs), which gives rise to allodynia or hyperalgesia. *In vitro*, the ubiquitin ligase Nedd4-2 is a negative regulator of VGSC α-subunits (Na_v), in particular $Na_v1.7$, a key actor in nociceptor excitability. We therefore studied Nedd4-2 in rat nociceptors, its co-expression with $Na_v1.7$ and $Na_v1.8$, and its regulation in pathology. Adult rats were submitted to the spared nerve injury (SNI) model of neuropathic pain or injected with complete Freund's adjuvant (CFA), a model of inflammatory pain. L4 dorsal root ganglia (DRG) were analyzed in sham-operated animals, seven days after SNI and 48 h after CFA with immunofluorescence and Western blot. We observed Nedd4-2 expression in almost 50% of DRG neurons, mostly small and medium-sized. A preponderant localization is found in the non-peptidergic sub-population. Additionally, 55.7 ± 2.7% and 55.0 ± 3.6% of Nedd4-2-positive cells are co-labeled with $Na_v1.7$ and $Na_v1.8$ respectively. SNI significantly decreases the proportion of Nedd4-2-positive neurons from 45.9 ± 1.9% to 33.5 ± 0.7% ($p < 0.01$) and the total Nedd4-2 protein to 44% ± 0.13% of its basal level ($p < 0.01$, $n = 4$ animals in each group, mean ± SEM). In contrast, no change in Nedd4-2 was found after peripheral inflammation induced by CFA. These results indicate that Nedd4-2 is present in nociceptive neurons, is downregulated after peripheral nerve injury, and might therefore contribute to the dysregulation of Na_vs involved in the hyperexcitability associated with peripheral nerve injuries.

Key words: Nedd4-2, neuropathic pain, voltage-gated sodium channels, $Na_v1.7$, $Na_v1.8$, dorsal root ganglion.

INTRODUCTION

Neuropathic pain affects a high proportion of the world population (Bouhassira et al., 2008) and originates from a maladaptive plasticity caused by a lesion in the somatosensory system (Woolf and Salter, 2000; Costigan et al., 2009). Clinically, it is associated with sensory dysfunctions referred as spontaneous pain, allodynia and hyperalgesia (Woolf and Decosterd, 1999). Convergent studies have shown that after a peripheral nerve lesion, ectopic activity, potentially accounting for pain symptoms, arises in injured and non-injured Aβ (normally non-nociceptive), Aδ and C-fibers (nociceptive) and in dorsal root ganglion (DRG) (Ma et al., 2003; Wu et al., 2001; Devor, 2009). Membrane hyperexcitability is thought to cause such abnormal generation of action potentials in DRG neurons (Amir et al., 2005) with the dysregulation of voltage-gated sodium channels (VGSCs) being the cornerstone of this regulation (Rush et al., 2007; Sheets et al., 2008).

Increased membrane ion permeability during action potential relies on the pore forming α-subunit (Na_v) of VGSCs, and its inhibition explains conduction blockade by local anesthetics (Catterall, 2000). Remarkably, the stability and internalization of ion channels are under the control of post-translational modifications, especially their ubiquitylation by E3 ubiquitin ligases driving their routing to degradation (Abriel and Staub, 2005). In particular the Nedd4 family of E3 proteins are potent regulators of channels, including Na_v (Abriel et al., 1999; Harvey and Kumar, 1999; van Bemmelen et al., 2004; Kabra et al., 2008). Most of the ten Na_v isoforms are expressed in DRG neurons and contribute to electrogenesis, with a specific expression for $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ in the peripheral nervous system (Black et al., 2002; Rush et al., 2007; Ho and O'Leary, 2011). Interestingly, mutations leading to loss or gain of $Na_v1.7$ function result in congenital insensitivity to pain or lead to severe familial pain disorders (Raouf et al., 2010). Mice with selective knock out of $Na_v1.7$, $Na_v1.8$

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Abbreviations: ATF-3, Activating Transcription Factor 3; CFA, complete Freund's adjuvant; DRG, dorsal root ganglion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK293, Human Embryonic Kidney cells; IB4, isolectin B4; IR, immunoreactivity; NGS, normal goat serum; RT, room temperature; SNI, spared nerve injury; VGSCs, voltage-gated sodium channels.

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and $Na_v1.9$ in DRG neurons have reduced pain sensitivity or diminished response to inflammatory pain (Nassar et al., 2004). These data suggest a key role of peripheral Na_vs in basal nociception, and also a possible impact of the regulation of nociceptor-specific Na_v in pathological pain. Various reports have indicated that inflammatory and neuropathic pain are associated with changes in $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ expression at both the mRNA and the protein levels in DRG neurons (Cummins and Waxman, 1997; Berta et al., 2008; Strickland et al., 2008; Thakor et al., 2009) but the mechanism leading to the selective alteration of some Na_v currents in neuropathic pain is still unclear (Berta et al., 2008). One theory posits the existence of refined mechanisms at the post-translational level leading to change in Na_v function or trafficking. In this context Nedd4-2, a well-described Nedd4 member, can interact with all Na_vs expressed in DRG neurons (except $Na_v1.9$) via their C-terminal PY motive. Indeed, *Xenopus oocytes* exogenously coexpressing Nedd4-2 with $Na_v1.2$, 1.7 and 1.8 present a reduction of their respective currents (Fotia et al., 2004). However, despite the apparent importance of Na_v regulation by Nedd4-2, the expression of Nedd4-2 in the nociceptive pathway has not been studied so far.

The aim of the present study is first to investigate *in vivo* the expression of Nedd4-2 in rat DRG and characterize its localization in the different subpopulations of primary sensory neurons using specific markers. In addition, we have assessed Nedd4-2 modulation in rodent models of neuropathic and inflammatory pain. Our results indicate that Nedd4-2 immunoreactivity (IR) is present in small diameter nociceptive neurons together with $Na_v1.7$ and $Na_v1.8$ and that this expression is down-regulated after nerve injury.

EXPERIMENTAL PROCEDURES

Surgery

All procedures were approved by the Committee on Animal Experimentation for the Canton of Vaud, Switzerland, in accordance with Swiss Federal Law on Animal Welfare and guidelines of the International Association for Study of Pain (IASP) (Zimmermann, 1983). We used the spared nerve injury (SNI) model of neuropathic pain as previously described (Decosterd and Woolf, 2000). Briefly, adult Sprague–Dawley rats were deeply anesthetized using 1.5% isoflurane and, after exposure of the sciatic nerve, the common peroneal and tibial nerves were ligated with 5.0 silk sutures and transected while the sural nerve was left intact. Muscle and skin were closed in two distinct layers with 5.0 silk thread and wound clips. Sham surgery was performed similarly, although without nerve damage, as the control condition. Eight animals were used for SNI ($n = 4$ for immunolabeling and $n = 4$ for Western blot) and a similar group of eight rats was used for sham surgery.

For experiments with complete Freund's adjuvant (CFA), rats were injected with 50 µl of CFA (Sigma, St. Louis MO, USA, $n = 4$) or NaCl 0.9% ($n = 4$), in the dorsal part of the left hindpaw, under isoflurane anesthesia (1.5% isoflurane). Animals were kept for 48 h and tissues dissected at this time-point for further analysis (Nagakura et al., 2003).

Immunohistochemistry

One week after the SNI surgery or 48 h after CFA injection, animals were lethally anesthetized with sodium pentobarbital and transcardially perfused with saline, followed by paraformaldehyde 4% in PBS. L4 DRGs (Hammond et al., 2004) were dissected and post-fixed at 4 °C for 90 min and then transferred in 20% sucrose in PBS overnight. The following day, tissues were mounted in cryoembedding fluid (Tissue-Tek; Sakura Finetek, Zoeterwoude, Holland). Then samples were frozen, cryosectioned in 12-µm thick sections and thaw-mounted onto slides.

Nedd4-2 was revealed using a specific rabbit anti-Nedd4-2 antibody (Nedd4-2, 1:100, generously provided by Olivier Staub, Department of Pharmacology and Toxicology, University of Lausanne, Switzerland). For the colocalization experiments, antibodies were as follows: mouse anti-peripherin (Peripherin; 1:500, Chemicon International, Billerica, MA, USA), mouse anti-neurofilament 200 (NF200, 1:400, Sigma, St. Louis, MO, USA), rat anti-Substance P (1:400, BD Bioscience, Basel, Switzerland), mouse anti-$Na_v1.7$ (1:100, Neuromab, Davis, CA, USA), mouse anti-$Na_v1.8$ (1:100, Neuromab, Davis, CA, USA), rabbit anti-Activating Transcription Factor 3 (ATF-3, 1:200, Santa Cruz Biotechnology, Heidelberg, Germany). Secondary antibodies were as follows: Cy3-conjugated anti-rabbit (1:400, Jackson ImmunoResearch, Suffol, UK) for Nedd4-2, Cy3-conjugated anti-mouse (1:300, Jackson ImmunoResearch) for $Na_v1.7$, FITC-conjugated anti-rabbit (1:200, Jackson ImmunoResearch, Suffol) for ATF-3, FITC-conjugated anti-rat (1:200, Jackson ImmunoResearch, Suffol) for Substance P, Alexa 488 anti-rabbit (1:500, Molecular Probes, Basel, Switzerland) for Nedd4-2 and Alexa 488 anti-mouse (1:1000, Molecular Probes) for peripherin, NF200, $Na_v1.8$. Non-peptidergic neurons were stained using biotinylated griffonia simplicifolia Isolectin B4 (IB4) (1:100, Vector Laboratories, Burlingame, CA) followed by AMCA-conjugated streptavidin (1:50; Jackson ImmunoResearch). Standard protocols for fluorescent immunohistochemistry were used. Sections of DRGs were blocked for 30 min at room temperature (RT) with 10% normal goat serum (NGS) and 0.3% PBS 1X-Triton X-100. Primary antibodies were diluted in 5% NGS and 0.1% PBS 1X-Triton X-100, and incubated on sections overnight at 4 °C. For ATF-3/Nedd4-2 dual labeling, the sequence of the protocol started with the primary and secondary incubations for ATF-3 followed by primary and secondary probing for Nedd4-2 (Pertin et al., 2005; Fukuoka et al., 2012). Control experiments were performed to rule out the possibility of a nuclear presence of Nedd4-2. Slides were washed in PBS and then incubated at RT with the corresponding secondary antibody or AMCA-conjugated streptavidin diluted in NGS 1% and PBS 1X-Triton X-100 0.1% for 90 min. Slides were washed in PBS and mounted in Mowiol medium (Calbiochem, Gibbstown, NJ).

Pictures and counting

Fluorescence was detected using an epifluorescent microscope (AxioPlan and AxiVision, Carl Zeiss, Feldbach, Switzerland). Images were taken at 20× magnification, with the same parameters used between experimental conditions, saved as TIFF files and then juxtaposed as one picture using Photoshop CS4 software (11.0, Sun Microsystems, Redwood City, CA) in order to reconstruct a complete DRG. The same parameters for image capture were used between experimental conditions and ganglia from four animals were analyzed per condition. Mean cell counts from each animal were the average of four sections selected 60 µm apart. The first slide was randomly selected and the three next ones were chosen every five slides from the series of consecutive cut sections. In all conditions, only neurons in which the nucleus was visible were counted. The observer was blinded to experimental groups. Counts of

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labeled neuronal profiles were expressed as a percentage of the total number of labeled and unlabeled neuronal profiles. Neuronal cross-sectional areas were measured in μm^2 and the mean gray value of each cell was recorded based on mean pixel intensity using ImageJ software (1.42, National Institute of Mental Health, Bethesda, MD, USA). Groups for cell area were as follows: 0–600 μm^2 for small neurons, 600–1200 μm^2 for medium-sized neurons and >1200 μm^2 for large neurons (Harper and Lawson, 1985; Noguchi et al., 1993).

Threshold of detection for IR-positive cells

Positively labeled cells were identified on acquired digital images by the experimenter. The accuracy of detection was verified for each condition by determining the signal/background threshold as follows (King et al., 2009). Ten pictures were randomly chosen in images libraries of four independent markers (Nedd4-2, $Na_v1.7$, peripherin and NF-200). Background intensities were measured and averaged. The final threshold of mean gray values was calculated by adding two standard deviations, giving a value of 18. Twenty out of 718 Nedd4-2-positive cells (sham) and 42 out of 616 positive cells (SNI) were below the detection threshold. For $Na_v1.7$ counting, all positive cells were above the detection threshold.

Cell transfection

Human Embryonic Kidney cells (HEK293) were cultured in DMEM (Gibco, Life Technologies, Zug, Switzerland) supplemented with 10% fetal bovine serum, 0.2% glutamine and gentamicin (20 mg/mL) at 37 °C in a 5% CO_2 incubator. For control of antibodies, cells were transfected, using calcium phosphate, with 0.8 µg of truncated human Nedd4-2 cDNA (without the amino-terminal C2 domain) cloned into pcDNA3.1 (generously provided by Olivier Staub, Department of Pharmacology and Toxicology, Lausanne, Switzerland) or 1 µg of $Na_v1.7$ cDNA cloned into pCIN5h and provided by Dr. Simon Tate (Convergence Pharmaceuticals, Cambridge, UK). Protein extraction was performed 48 h after transfection.

Western blotting

Animals were sacrificed 7 days after Sham or SNI surgery, or 48 h after CFA injection. L4 and L5 DRGs were quickly dissected and kept at −80 °C until use. HEK293 transfected cells were detached using dissociation buffer (Invitrogen, Life Technologies), centrifuged at 2000*g* for 2 min at RT and supernatants were removed. Homogenization of DRGs or HEK cells was done in 100 mM Tris–HCl (pH 6.8), SDS 2%, glycerol 20%, NaCl and complete protease inhibitor cocktail tablets (Roche, Basel, Switzerland). Samples were centrifuged at 14,000 rpm for 20 min at 4 °C and proteins in the supernatants were quantified using Bradford assays. Protein samples (15–20 µg) were separated by SDS–PAGE and transferred onto PVDF membranes (BioRad, Hercules, CA). Blots were blocked with non fat dry milk 5% for 30 min at RT and then incubated overnight at 4 °C with the appropriate antibody: rabbit anti-Nedd4-2 (1:100) (Flores et al., 2005), mouse anti-$Na_v1.7$ (1:400, Neuromab) or mouse anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:500,000, Abcam, Cambridge, UK). These blots were further incubated with horseradish peroxydase-conjugated secondary antibody anti-mouse or anti-rabbit (1:2000, Dako, Heverlee, Belgium), developed in Super Signal Solution (Pierce, Rockford, IL, USA) and revealed with LAS-4000-Mini Fujifilm (Bucher Biotec, Basel, Switzerland). Pixel intensities were quantified with ImageJ. Results were expressed as the ratio of the signal of interest over sham after normalization by GAPDH loading control.

Statistics

Data are represented as mean ± SEM. Comparisons between groups were performed using Student's *t* test or a one-way ANOVA followed by Dunnett's Multiple Comparison Test for Fig. 3F. Statistical analyses were done with JMP statistical software (5.01, SAS institute, Cary, NC). Differences were considered significant at *p*-values below 0.05.

RESULTS

Nedd4-2 is present in nociceptive neurons

Nedd4-2 IR is widely distributed in rat DRG neurons (Fig. 1A). Western blot analysis (Fig. 1B) reveals one major band in DRG at approximately 120 kDa corresponding to the endogenous form of Nedd4-2 (lane 1). In HEK293 cells, the endogenous form of Nedd4-2 corresponds to a slightly lower band (~115 kDa), in line with species differences (van Bemmelen et al., 2004; Rougier et al., 2005). In DRG, we inconstantly observed one additional band between 100 and 120 kDa, which is known to be a splice variant (Itani et al., 2003; Hryciw et al., 2004) (see Figs. 3A and 5B). As a positive control, the analysis of HEK293 cells transfected with a truncated form of human Nedd4-2 cDNA showed a robust protein level at 100 kDa, in line with the size of the construct (Kamynina et al., 2001).

We thoroughly explored the molecular identity of Nedd4-2-immunopositive DRG neurons using dual immunofluorescence (Fig. 2). The six sets of counting performed throughout our study gave overall percentages of Nedd4-2-expressing neurons ranging

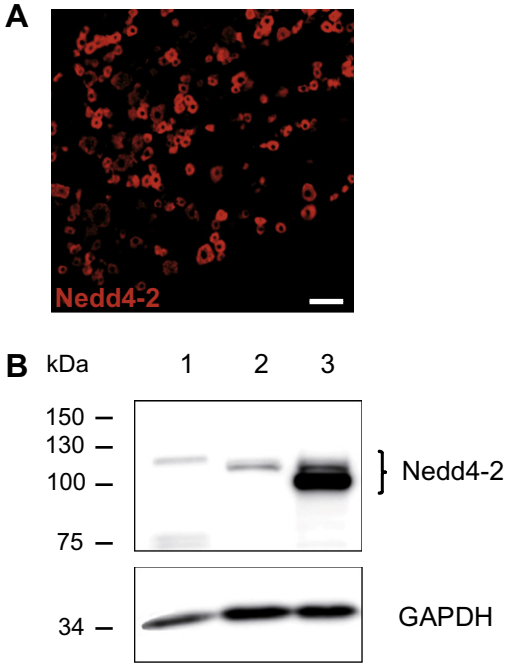

Fig. 1. Protein expression of Nedd4-2 in rat DRG. (A) Nedd4-2-immunoreactivity (IR) in rat L4 DRG (scale bar = 100 µm). (B) Western blot analysis of Nedd4-2. Lane 1: rat L4 DRG; lane 2: HEK293 cells; lane 3: Nedd4-2 transfected HEK293 cells; GAPDH was used as a loading control.

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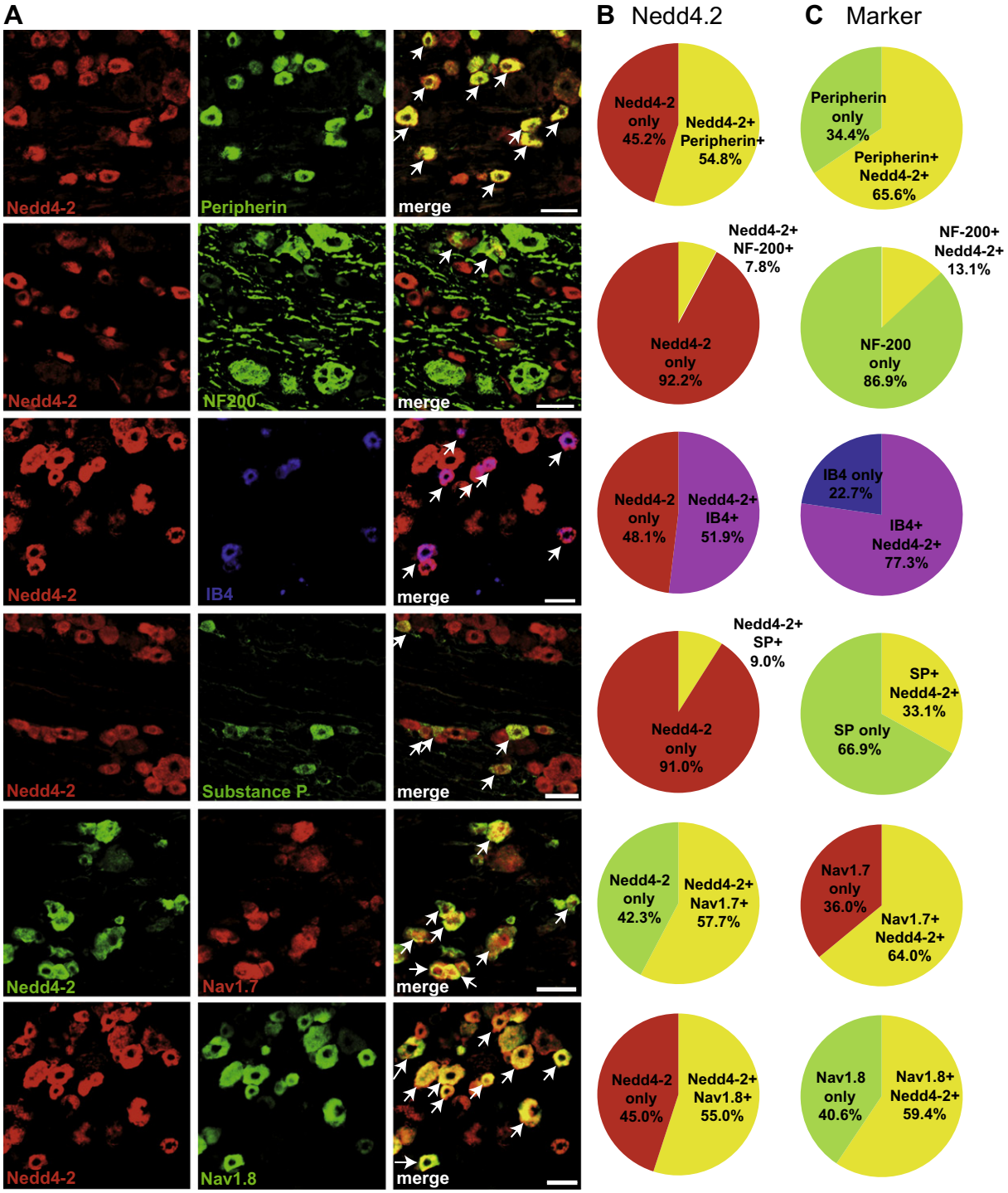

Fig. 2. Nedd4-2 is mainly present in nociceptive DRG neurons and colocalizes with the sodium channels $Na_v1.7$ and $Na_v1.8$. (A) Representative double immunofluorescence for Nedd4-2 (first column) with the markers peripherin, NF-200, IB4, Substance P, or with $Na_v1.7$ and $Na_v1.8$ (second column) and merged images (third column, arrows) in rat DRG neurons (sham group). (B) Quantitative analysis of Nedd4-2-IR with the different markers. Among all Nedd4.-2-positive neurons, 54.8 ± 3.8% co-stained for peripherin, 7.8 ± 2.7% for NF-200, 51.9 ± 2.2% for IB4, 9.0 ± 1.2% for Substance P, 57.7 ± 2.7% for $Na_v1.7$ and 55.0 ± 3.6% for $Na_v1.8$. (C) Among the following markers, many co-stained with Nedd4-2: peripherin (65.6 ± 3.2%), NF-200 (13.1 ± 3.7%), IB4 (77.3 ± 4.6%), Substance P (33.1 ± 2.8%), $Na_v1.7$ (64.0 ± 2.9%) and $Na_v1.8$ (59.4 ± 4.9%). Data are presented as mean ± SEM. $n = 4$. Scale bar = 50 μm.

from 43.3 ± 2.6% to 49.6 ± 0.9% of total neurons ($n = 4$). Peripherin, an intermediate filament selective for small sensory neurons presumably nociceptive, is found in about half of neurons with Nedd4-2-IR (Fig. 2B) and conversely Nedd4-2-IR is present in a large majority of peripherin-positive neurons (Fig. 2C). Nedd4.2 is weakly expressed in the NF200-postive large myelinated fiber cell population. Within the subpopulations of

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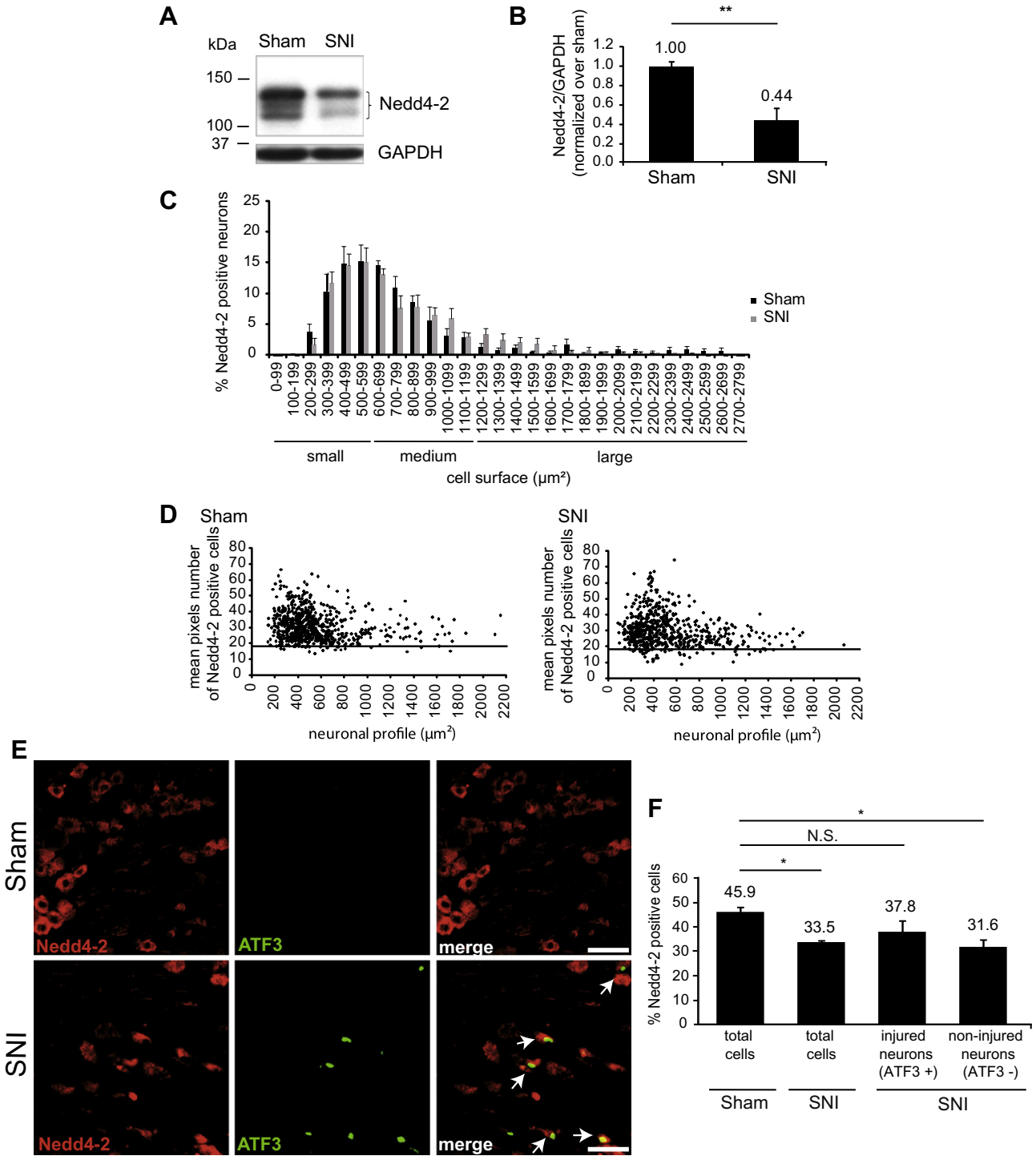

Fig. 3. Peripheral nerve injury decreases Nedd4-2 in DRG neurons. (A) Representative Western blot analysis one week after sham or SNI surgeries. (B) Quantification of Nedd4-2 protein content indicates a downregulation of Nedd4-2 in L4 DRG after SNI (quantifications are expressed as ratio over sham signal after normalization to GAPDH signal). (C) Distribution of cross-sectional area of Nedd4-2-positive neurons in the L4 DRG; 0–600 µm^2 indicates small DRG neurons, 600–1200 µm^2 medium DRG neurons, >1200 µm^2 represents the large DRG neurons (Noguchi et al., 1993). 507 Nedd4-2-positive neurons were measured in the sham group, 438 in the SNI group. (D) Representation of mean pixel number (intensity) of all Nedd4-2-IR profiles according to their size. The horizontal line at 18 represents the threshold between positive and negative neurons. (E) Representative immunofluorescence showing Nedd4-2 (red) in both neurons expressing ATF-3 (green), a marker of injured neurons, and ATF-3 immuno-negative cells in L4 DRG. (F) Quantification of Nedd4.2-IR one week after SNI or sham surgery, in injured/ATF-3-positive profiles. Results are expressed in mean ± SEM, $n = 4$ animals in each group for all panels. $^{**}p < 0.01$, $^{*}p < 0.05$. N.S., non-significative. Student's t test. One-way ANOVA followed by Dunnett's Multiple Comparison Test for Fig. 3F. Scale bar = 50 µm.

small neurons, Nedd4-2-positive cells are mainly represented in the non-peptidergic neurons (IB4 positive) while less than 10% of Nedd4-2 immunoreactive cells are positive for the neuropeptide Substance P.

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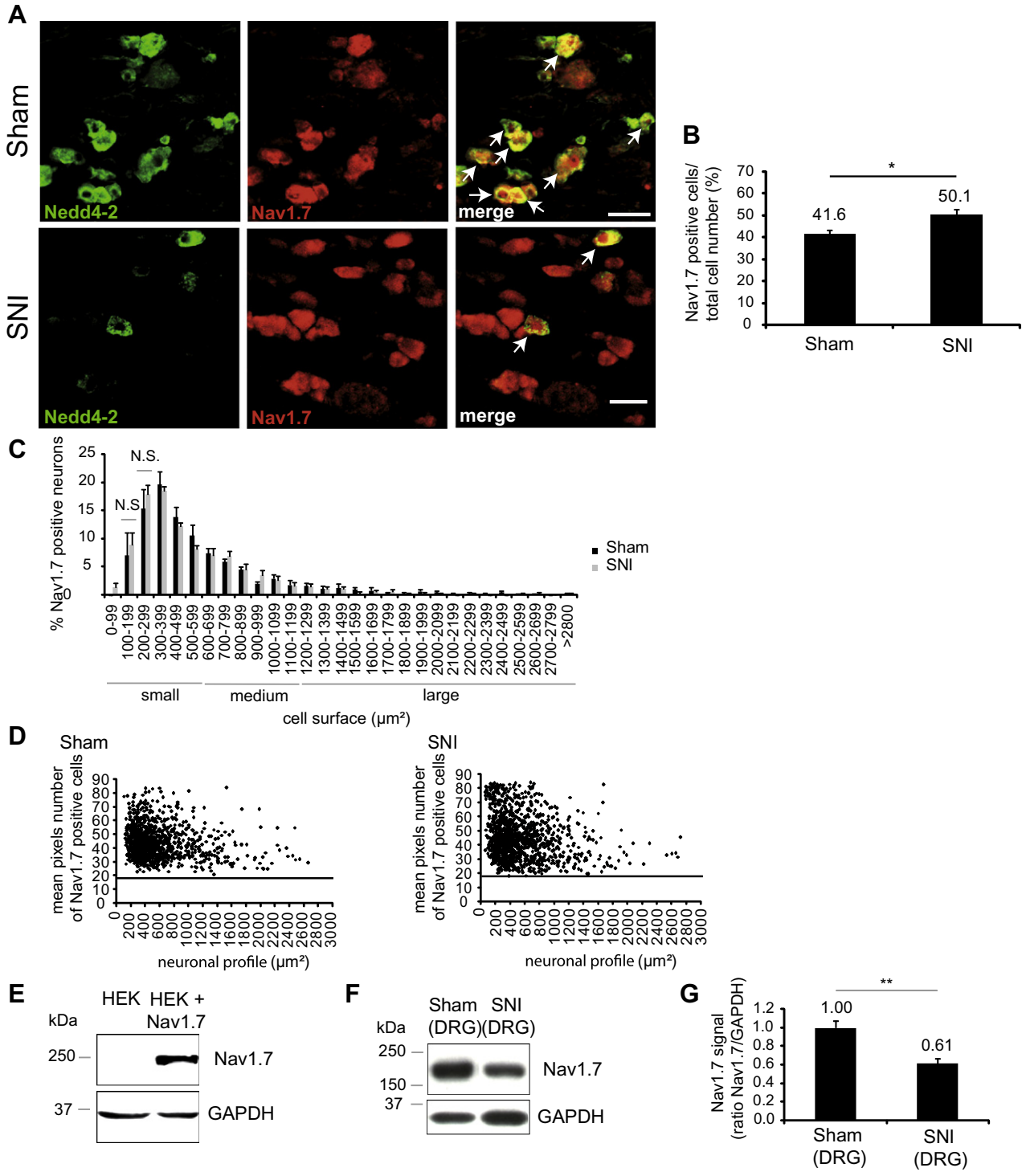

Fig. 4. Nedd4-2-IR and $Na_v1.7$ expression one week after SNI. (A) Immunofluorescence showing the colocalization (right panel, arrows) between Nedd4-2 immunosignal (green, left panel) and $Na_v1.7$ (red, middle panel) in rat lumbar DRG. Scale bar = 50 µm. (B) Quantification of $Na_v1.7$-immunoreactive cells. (C) Cell-size of $Na_v1.7$-IR profiles in L4 DRG, expressed as the percentage of $Na_v1.7$-positive cells in total cells. 0–600 µm² indicates small neurons, 600–1200 µm² medium neurons, > 1200 µm² represents large neurons. 1040 $Na_v1.7$-positive neurons were measured in the sham group and 1304 in the SNI group. (D) Representation of mean pixel number of all $Na_v1.7$-positive cells according to their size. The horizontal line at 18 represents the threshold between $Na_v1.7$-positive and -negative neurons. (E) Control of $Na_v1.7$ antibody by Western blot analysis in HEK293 cells. The first lane represents native HEK cells and the right lane HEK cells transfected with $Na_v1.7$ cDNA. GAPDH was used as a loading control. (F) Representative Western blot of $Na_v1.7$ one week after sham and SNI surgery DRG. GAPDH was used as a loading control. (G) Quantification of Western blot analysis. A significant decrease of $Na_v1.7$ expression was observed when compared to sham. Quantifications are expressed as ratio over sham after normalization to GAPDH. Results are expressed in mean ± SEM and $n = 4$ animals in all panels. $^{*}p < 0.05$, $^{**}p < 0.01$, Student's t test.

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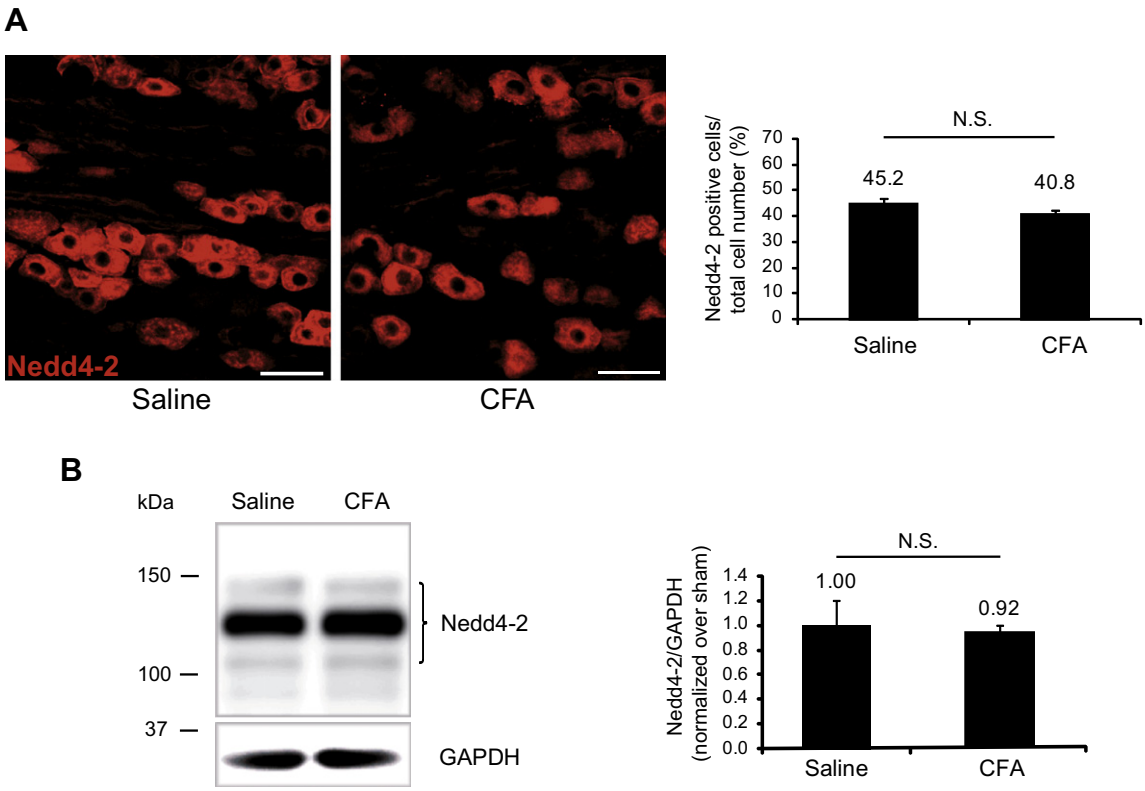

Fig. 5. Peripheral inflammation does not induce changes in Nedd4-2 expression in the DRG. (A) Representative immunofluorescence from L4 DRG showing the absence of significative difference in Nedd4-2 signal between saline or CFA-treated rats. Right bar histogram shows the quantification of Nedd4-2-immunoreactive profiles in total neuronal profiles. (B) Western-blot analysis of rat L4 DRG after saline or CFA administration. Quantifications are expressed as ratio over GAPDH signal, normalized over sham signal. Results are expressed as mean ± SEM, $n = 4$ animals in each group. N.S., non-significative, Student's t test. Scale bar = 50 μm.

Together, these results indicate that Nedd4-2 is expressed in DRG neurons and is predominantly localized in small diameter sensory neurons, which include many nociceptive neurons.

Nedd4-2-IR is decreased after SNI

In order to test the hypothesis that Nedd4-2 plays a role in the hyperexcitability associated with neuropathic pain in rats, we explored the regulation of its expression following peripheral nerve injury. Using Western blot analysis of L4 DRG (Fig. 3A and B), we observed a decrease of about 56% in Nedd4-2 protein content after SNI. Similar results were observed using immunofluorescence on DRG (Fig. 3E and F): quantification indicated a decrease in the number of Nedd4-2-IR cell profiles after SNI. In addition, we evaluated whether Nedd4-2 signal could be shifted to another DRG neurons subpopulation after nerve injury (Fig. 3C). The distribution of Nedd4-2-positive neurons in DRG cells of different sizes confirms an expression mainly in small neurons ($< 600\ \mu m^2$), but no significant difference was detected between sham and SNI groups. The reduction of Nedd4-2-IR observed after SNI is not associated with a decrease of mean pixel intensity calculated for individual neurons (Fig. 3D), but rather with a reduced number of Nedd4-2-expressing neurons (Fig. 3F).

We therefore investigated whether the downregulation of Nedd4-2-IR occurs in injured DRG neurons (injured afferents of the tibial and peroneal nerves) after SNI, which are known to be positive for the transcription factor ATF-3 (Tsujino et al., 2000; Miyoshi et al., 2011). As anticipated, the proportion of immunoreactive neurons for ATF-3 in SNI rats is higher than in the sham group (34.8 ± 2.8% as compared to 4.8 ± 0.2%), yet a detailed analysis shows that downregulation of Nedd4-2 occurs in both injured (ATF positive) and the remaining non-injured (ATF negative) neurons present in the DRG (Fig. 3F). This suggests that neuronal deterioration and in turn the previously reported neuronal death following nerve section (Tandrup et al., 2000; McKay Hart et al., 2002) is unlikely to account for the reduction of Nedd4-2 IR, in line with the reported absence of neuronal death in neuropathic pain without axonal injury (Schaeffer et al., 2010).

Nedd4-2-IR colocalizes with sodium channels $Na_v1.7$ and $Na_v1.8$

The regulation of ion channels by Nedd4-2, in particular the voltage-gated sodium channels $Na_v1.7$ and $Na_v1.8$, implies their coexpression in the same DRG neurons. In line with this hypothesis, strong colocalizations between Nedd4-2 and $Na_v1.7$ (Figs. 2 and 4A) or $Na_v1.8$ (Fig. 2) were found. More than 60% of $Na_v1.7$-positive neurons

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co-expressed Nedd4-2, a value that dropped concomitantly with Nedd4-2 downregulation after SNI (64.0 ± 2.9% to 40.1 ± 3.0% in sham and SNI groups respectively, $p < 0.01$, $n = 4$ in each group). A significant increase in the number of $Na_v1.7$ immunoreactive neuronal profiles is observed after SNI as compared to sham (Fig. 4B), without any significant change in the distribution of $Na_v1.7$-IR cross-sectional area (Fig. 4C). The enrichment in $Na_v1.7$-IR is mostly observed in small neurons (0–600 μm^2) and a simultaneous increase in mean pixel intensity is observed in individual cells of this category after SNI (Fig. 4D). Remarkably, Western blot quantification shows a decrease in $Na_v1.7$ content in DRG after SNI (Fig. 4F–G).

Many $Na_v1.8$-IR cells express Nedd4-2 (Fig. 2A–C) in a proportion that is not altered by SNI (59.4 ± 4.9% and 59.6 ± 5.3% in sham and SNI groups respectively, $n = 4$). Conversely, 55.0 ± 3.6% of Nedd4-2-IR profiles showed $Na_v1.8$-IR, but this proportion was significantly decreased to 38.6 ± 4.4% after SNI ($p < 0.05$, $n = 4$). This parallels the known downregulation of $Na_v1.8$ after peripheral nerve injury, which is here confirmed by the decrease of cells expressing $Na_v1.8$ in DRG: 46.9 ± 5.2% of total DRG neuronal profiles showed $Na_v1.8$-IR from control rats compared with 21.1 ± 1.7% of DRG neuronal profiles from SNI animals ($p < 0.01$, $n = 4$).

Nedd4-2 IR is not altered by peripheral inflammation

We further tested the hypothesis that Nedd4-2 might be regulated after peripheral inflammation (Fig. 5). As shown in (Fig. 5A and B) 48 h following intraplantar injection of CFA, neither the density of Nedd4-2-positive neurons nor the global Nedd4-2 content was modified.

DISCUSSION

In the present study, we characterized the expression of Nedd4-2 in primary sensory neurons and showed its downregulation after a peripheral nerve lesion. Using immunofluorescence and Western blot, we first established the presence of Nedd4-2-IR in DRG neurons and its main localization in small-diameter neurons. Furthermore, after SNI, we found a significant decrease in Nedd4-2 content in DRG neurons. Finally we demonstrated that the expressions of $Na_v1.7$ and $Na_v1.8$ strongly co-localize with Nedd4-2-IR in the basal condition and after peripheral nerve injury.

The demonstration of a colocalization between Nedd4-2 and $Na_v1.7$ or $Na_v1.8$ immunoreactivities was the first crucial step before postulating an involvement of Nedd4-2 in the regulation of Na_v after peripheral nerve injury. In rat DRG, we found that a large proportion of neurons were immunoreactive for Nedd4-2, a proportion that further increased when we considered the sole population of small neurons that express peripherin. This result, together with the low percentage of Nedd4-2-positive cells in myelinated NF200 immunoreactive neurons (presumably non-nociceptive except the Aδ subpopulation), suggests that Nedd4-2 may have a role to play in the physiology of nociceptive neurons. In addition, the further enrichment of Nedd4-2 in IB4-positive nociceptive neurons (77.3 ± 4.6%) indicates a putative specific role of Nedd4-2 in the excitability of non-peptidergic nociceptors, a cell population described as having longer duration action potentials and expressing a high density of $Na_v1.8$ (Stucky and Lewin, 1999). The marked downregulation of Nedd4-2-IR in DRG neurons following peripheral nerve injury suggests changes in ion channel trafficking and the possible role of this in neuropathic pain is a promising subject for future study. After nerve injury, the reduced proportions of Nedd4-2-positive neurons were not significantly different between injured (ATF-3 positive) and non-injured (ATF-3 negative) DRG neuronal populations. This might imply that Nedd4-2 downregulation contributes to Na_v turnover in both injured (axotomized) and non-injured adjacent neurons.

Sodium channels exist both at the plasma membrane and in intracellular pools (Schmidt et al., 1985; Ritchie et al., 1990). Multiple and complex mechanisms contribute to the forward trafficking of sodium channels – and other voltage-gated ion channels – from the intracellular pool to their subsequent functional insertion in the plasma membrane. In this process, insertion is counter-balanced by various post-translational modifications including ubiquitylation and consequent internalization of the channel (Jenkins and Bennett, 2001; Okuse et al., 2002; Garrido et al., 2003; Mohler et al., 2004; Lopez-Santiago et al., 2006), a phenomenon in which Nedd4-2 has been implicated in transfected cells (Fotia et al., 2004). In SNI, the Nedd4-2 decrease may influence this balance, possibly leading to an accumulation of Na_v at the cell membrane while the total quantity of sodium channels in neurons may remain stable. This mechanism might explain discrepancies that have been reported between the apparent rate of Na_v synthesis and the observed functional current, as in the case of TTX sensitive Na_v isoforms (Berta et al., 2008). In accordance with previous studies on $Na_v1.7$ transcriptional expression (Raymond et al., 2004; Berta et al., 2008), we found a significant reduction in total $Na_v1.7$ protein after SNI. Nevertheless, the number of $Na_v1.7$-IR neurons was increased after SNI. This discrepancy might originate from a redistribution of $Na_v1.7$ protein to a different neuronal compartment more easily accessible to the antibody, such as plasma membrane. It is also possible that following peripheral nerve injury $Na_v1.7$ is upregulated but redistributed from the soma of sensory neurons toward fibers resulting in apparent simultaneous signal reduction in the cell bodies and increase in the total DRG protein content.

Further investigations should be conducted in order to distinguish the membrane and intracellular pools of $Na_v1.7$ using electrophysiological and biochemical approaches. $Na_v1.7$ has so far never been investigated in DRG using patch clamp techniques due to the difficulty to selectively isolate its currents. Interestingly, however, $Na_v1.7$ specific blockers have recently been developed and will allow $Na_v1.7$ exploration (Schmalhofer et al.,

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2008; Liu et al., 2012). Furthermore, biochemical strategies might also be employed to quantify the internalization and membrane targeting of Na_v in neuropathic pain and the importance of the specific interaction with Nedd4-2 in this process.

The strong downregulation of $Na_v1.8$, in line with many other studies but in apparent contradiction of our hypothesis, would suggest that a transcriptional downregulatory mechanism predominates regardless of the inhibition by Nedd4-2 of channel internalization. In addition, it was already proposed that the downregulation of $Na_v1.8$ mRNA and protein in DRGs is due to a redistribution of this channel along the axons of injured (Thakor et al., 2009) or uninjured nerves (Gold et al., 2003). Nedd4-2 downregulation in the DRG might also impact $Na_v1.8$ expression at the membrane along the axon and further studies are needed to answer this question.

Nedd4-2 is not the only potential post-translational regulator of Na_v. An interaction between p11 (from the S100 protein family) and $Na_v1.8$ has been reported to facilitate $Na_v1.8$ sorting toward the cell membrane (Okuse et al., 2002). Besides, ankyrin interacts with and upregulates $Na_v1.5$ in cardiac cells (Mohler et al., 2004) as well as $Na_v1.2$ and $Na_v1.6$ at nodes of Ranvier (Jenkins and Bennett, 2001; Garrido et al., 2003). In addition, $Na_v\beta$-subunits fulfill important regulatory functions. In particular, the β2-subunit was shown to modulate mRNA and protein expression of various Na_v (Lopez-Santiago et al., 2006) and is increased after nerve injury (Pertin et al., 2005). Finally, protein kinases such as PKA or PKC also modulate VGSC, with PKA increasing $Na_v1.8$ and decreasing $Na_v1.7$ currents while PKC decreases $Na_v1.8$ and $Na_v1.7$ currents (Vijayaragavan et al., 2004). Nedd4-2 interacts specifically via one of its WW domains (protein–protein interaction modules) with a PY motif situated in the COOH termini of Na_v or ENaC (Harvey et al., 1999; Rougier et al., 2005). Previous studies have established the connection between Nedd4-2 and the Na_v *in vitro* (Fotia et al., 2004; van Bemmelen et al., 2004; Rougier et al., 2005). In cardiac cells, $Na_v1.5$ can be downregulated by Nedd4-2 (Abriel et al., 2000; van Bemmelen et al., 2004) implying a probable modulation of cardiac excitability. With the exception of $Na_v1.4$ and $Na_v1.9$, all Na_v contain the specific PY motif suggesting an interaction with Nedd4-2. These include $Na_v1.6$, whose mRNA is the third most abundant among sodium channels in the DRG (Berta et al., 2008); this channel has recently been reported to be modulated by Nedd4-2 (Gasser et al., 2010). Additionally, $Na_v1.2$, mainly present in the central nervous system, has been shown to be downregulated when associated with Nedd4-2 (Fotia et al., 2004; Rougier et al., 2005). In addition to Na_v, Voltage-gated K+ channels play major roles in modulating electrical excitability in neurons. For instance, KCNQ2/3/5contain a PY motif and is subjected to Nedd4-2 dependent downregulation in a *Xenopus oocyte* expression system (Ekberg et al., 2007; Pongs, 2008; Bongiorno and Poronnik, 2011). This regulation and modulation of K+ channels might also be a key point in the excitability generated after a peripheral nerve injury.

Finally, besides its expression level, Nedd4-2 is regulated by a direct phosphorylation as well (Debonneville et al., 2001; Snyder, 2009). In particular, serum- and glucocorticoid kinase 1 (Debonneville et al., 2001) increases ENaC cell-surface expression through a negative regulation of Nedd4-2 (Alvarez et al., 1999). These findings imply a posttranslational regulation of Nedd4-2 playing therefore a role in Na_v modulation. Upstream regulatory mechanisms of Nedd4-2 might therefore represent other perspectives to explore in the context of peripheral nerve injuries.

CONCLUSION

In summary, we have demonstrated *in vivo* the presence of the ubiquitin ligase Nedd4-2 in the rat DRG. Moreover meticulous analyses of the immunoreactive cell populations showed its presence mainly in small nociceptive neurons, especially the non-peptidergic neurons. We also colocalized Nedd4-2 with $Na_v1.7$ and $Na_v1.8$. In a model of peripheral nerve injury, the decrease of Nedd4-2-positive neurons suggests a putative role in altered Na_v turnover, especially $Na_v1.7$, which could contribute to hyperexcitability. Future studies will shed light on the exact molecular impact of Nedd4-2 on Na_v in nociceptors and pathological pain.

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