SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

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DIPARTIMENTO (in caso di borsa per soggiorno all'estero specificare l'ente presso cui si è svolta la ricerca): dipartimento di Neuroscienze e area del farmaco e salute del bambino (Neurofarba)

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TIPOLOGIA DI BORSA RICEVUTA: Borse di Studio per progetti di ricerca in ambito farmacologico bandite dalla SIF grazie al contributo incondizionato di MSD Italia

TIPOLOGIA DI RELAZIONE (es.: metà periodo o finale): metà periodo

TITOLO DELLA RELAZIONE: Study of a functional interaction between the RNA binding protein HuD and mTOR in a model of neuropathic pain

RELAZIONE:

Background:

Neurons are arguably the most complex cells in the mammalian organism, which implies that they require a particularly diversified proteome. In addition, neurons have to store previous input by modulating their synaptic strength, which requires mechanisms that quickly adapt the proteome at the synapse to the changing needs. To meet these requirements, neurons make extensive use of post-trascriptional gene regulation. These regulatory events are orchestrated by trans-acting factors, a group of regulatory molecules comprising ELAV/RNA-binding proteins that aggregate onto mRNAs and form messenger ribonucleoprotein (mRNP) complexes (Lukong et al. 2008). The regulation of nociceptor physiology at the post-trascriptional level is more complex than has been previously recognized and that a greater knowledge of how gene expression is regulated within localized regions of these neurons, such as at the peripheral terminals, may represent a fruitful avenue for the exploration of novel therapeutic opportunities. Among all the RBPs expressed in neurons, the ELAV-like member HuD is one of the earliest markers of the neuronal phenotype and, plays important roles in several functions of neuron. Although, HuD has been reported to be increased in the DRG and sciatic nerve of animals with a trauma-induced neuropathic pain (Anderson et al., 2003, Yoo et al., 2013) and at spinal and supraspinal levels in antiretroviral neuropathy (Sanna et al., 2014), its role into nociception is entirely unknown, as well as the underlying molecular mechanisms and interactions. Thus, in the first part of the project, on basis of the role of ELAV-like HuD protein in axonal and neuronal recovery after nerve damage, we have characterized the contribution of this RNA binding protein, on peripheral nociceptor sensitivity and

regeneration, in animals which were suffering from neuropathic pain that was induced by spared nerve injury (SNI).

Materials and methods

Animals

Male CD1 mice (20-22 g) from the Harlan Laboratories (Bresso, Italy) breeding farm were used. Mice were randomly assigned to standard cages, with four to five animals per cage. The cages were placed in the experimental room 24 h before behavioural test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1 °C with a 12 h light/dark cycle, light on at 7 a.m. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee of the University of Florence, Italy, under license from the Italian Department of Health and in compliance with the European Communities Council directive of 24 November 1986 (86/609/EEC). All studies involving animals are reported in accordance with the ARRIVE guidelines for experiments involving animals.

Spared nerve injury (SNI)

Behavioral testing was performed before surgery to establish a baseline for comparison with postsurgical values. Mono-neuropathy was induced according to the method of Bourquin et al. Mice were anaesthetized with sodium pentobarbital (60 mg/kg i.p.). The right hind limb was immobilized in a lateral position and slightly elevated. Incision was made at mid-thigh level using the femur as a landmark. The sciatic nerve was exposed at mid-thigh level distal to the trifurcation and freed of connective tissue; the three peripheral branches (sural, common peroneal, and tibial nerves) of the sciatic nerve were exposed without stretching nerve structures. Both tibial and common peroneal nerves were ligated and transacted together. A microsurgical forceps with curved tips was delicately placed below the tibial and common peroneal nerves to slide the thread (5.0 silk, Ethicon; Johnson & Johnson Intl, Brussels, Belgium) around the nerves. A tight ligation of both nerves was performed. The sural nerve was carefully preserved by avoiding any nerve stretch or nerve contact with surgical tools. Muscle and skin were closed in two distinct layers with silk 5.0 suture. Intense, reproducible and long-lasting thermal hyperalgesia and mechanical allodynia-like behaviors are measurable in the non-injured sural nerve skin territory. The SNI model offers the advantage of a distinct anatomical distribution with an absence of co-mingling of injured and non-injured nerve fibers distal to the lesion such as the injured and not injured nerves and territories can be readily identified and manipulated for further analysis (i.e. behavioral assessment). The sham procedure consisted of the same surgery without ligation and transection of the nerves.

Behavioral testing

Animals were habituated to the experimental room and randomly assigned to each treatment group. Mice were investigated by observers blinded for treatment of the animals.

Von Frey test

Mechanical allodynia was measured by using Dynamic Plantar Anesthesiometer (Ugo Basile). The mice were placed in individual Plexiglas cubicles (8.5 cm L 3,4 cmH3,4 cm) on a wire mesh platform and allowed to acclimate for approximately 1 h, during which exploratory and grooming activity ended. After that, the mechanical stimulus was delivered to the plantar surface of the hind paw of the mouse from below the floor of the test chamber by an automated testing device. A steel rod (2 mm) was pushed with electronic ascending force (0–5 g in 35 s).When the animal withdrew its hind paw, the mechanical stimulus was automatically withdrawn and the force recorded to the nearest 0.1 g. Nociceptive response for mechanical sensitivity was expressed as mechanical paw withdrawal threshold. Each mouse served as its own control, the responses being measured both before and after administrations. PWT was quantified by an observer blinded to the treatment.

Hargreaves' plantar test

Thermal nociceptive threshold was measured using Hargreaves' device.18 Paw withdrawal latency in response to radiant heat (infrared) was assessed using the plantar test apparatus (Ugo Basile, Comerio, Italy). Each mouse was placed under a transparent Plexiglas box (7.0 × 12.5 cm2, 17.0 cm high) on a 0.6-cm-thick glass plate and allowed to acclimatize for 1–2 h before recording. The radiant heat source consisted of an infrared bulb (Osram halogen-bellaphot bulb; 8 V, 50 W) that was positioned 0.5 cm under the glass plate directly beneath the hind paw. The time elapsed between switching on the infrared radiant heat stimulus and manifestation of the paw withdrawal response was measured automatically. The intensity of the infrared light beam was chosen to give baseline latencies of 10 s in control mice. A cut-off of 20 s was used to prevent tissue damage. Each hindpaw was tested 2–3 times, alternating between paws with an interval of at least 1 min between tests. The interval between two trials on the same paw was of at least 5 min. Nociceptive response for thermal sensitivity was expressed as thermal paw withdrawal latency in seconds. All determinations were averaged for each animal.

Antisense oligonucleotide administration

To obtain the HuD silencing an antisense strategy was used since the available HuD constitutive knockout mouse shows some minor nerve development problems (Akamatsu et al., 2005) and developmental alteration of cortical connectivity (DeBoer et al., 2014), which would confound the comparison with a wild-type control animal. Moreover, aODN administration allows us to specifically downregulate gene expression in a reversible manner, as we have observed also in previous works (Galeotti et al., 1997; 2008). Phosphodiester oligonucleotides (ODNs) protected from terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were obtained from Tib Molbiol (Genoa, Italy). The aODN against HuD was the following: 5′-G*T*TCTGGAGCCTCATC*T*T-3′ where the asterisks indicate the phosphorotioate phosphate groups. Two 18 and 20mer fully degenerated ODNs (dODNs), where each base was randomly G, or C, or A, or T, were used as control treatment. aODNs and dODNs were preincubated at 37 °C for 30 min with an artificial cationic lipid (13 μM DOTAP, Sigma, Milan, Italy), to enhance both uptake and stability, before administration. To achieve protein knockdown, mice received a single i.t. injection every 24 h on day 1, 2 and 3 for a total of 3 injections.

Western blot analysis

Membrane homogenates (10-50 μg) were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes (90 min at 120 V) using standard procedures. Membrane were blocked in

PBST (PBS containing 0.1% Tween) containing 5% nonfat dry milk for 120 min. Following washings, blots were incubated overnight at 4° C with specific antibodies against HuD (1: 1000) and GAP43(1:1000). After being washed with PBS containing 0.1% Tween, the nitrocellulose membrane was incubated with goat anti-rabbit, anti-mouse horseradish peroxidase-conjugated secondary antisera (1:10,000) and left for 1 h at room temperature. Blots were then extensively washed according to the manufacturer's instruction and developed using enhanced chemiluminescence detection system. Exposition and developing time used was standardized for all the blots. Optical density measurements were performed by dividing the intensity of the bands by the intensity of the house-keeping protein ß-actin, used as loading control, at each time point. Measurements in control samples were assigned a relative value of 100%.

RNA Analysis

TRIzol (Invitrogen) was used to extract total RNA, and acidic phenol (Ambion) was used to extract RNA for RIP analysis. RT was performed using random hexamers and Maxima Reverse Transcriptase (Thermo Scientific), and real-time qPCR was done using gene-specific primers (Supplemental Experimental Procedures). RT-qPCR was performed using SYBR Green Master Mix (Kapa Biosystems) in an Applied Biosystems 7300 instrument.

Immunofluorescence

Mice were perfused transcardially with 0.9% NaCl. Lumbar spinal cord and dorsal root ganglia were isolated from adult male CD1 mice and fixed in 4% formalin. Sections were subjected to antigen retrieval in Na-citrate buffer (10mM, pH 6) for twenty minutes at 99° C. After preincubation in 5mg/ml BSA/0.3% Triton-X-100/PBS, sections were incubated overnight at 4°C with primary antibody at optimized working dilution. HuD (1:500), GAP43 (1:100), NF200 (1:100) and CGRP (1:100) antibodies were detected by Alexa-conjugated secondary antibodies (1:400). Sections were coverslipped using Vectorshield mounting medium with DAPI. A Leica DF 350 FX microscope with appropriate excitation and emission filters for each fluorophore was used to acquire representative images. Images were acquired with x 20 to x 40 objectives using a digital camera.

Results

Fig. 1 Selective up-regulation of HuD in dorsal root ganglia of spared nerve injury-mice

HuD mRNA levels increased at 3 days in the ipsilateral side of dorsal root ganglia, while the correspondent protein increased at 7 days post-crush (Fig.1A-B). Conversely no modulation of HuD was detected in the spinal cord (Fig. 1C).

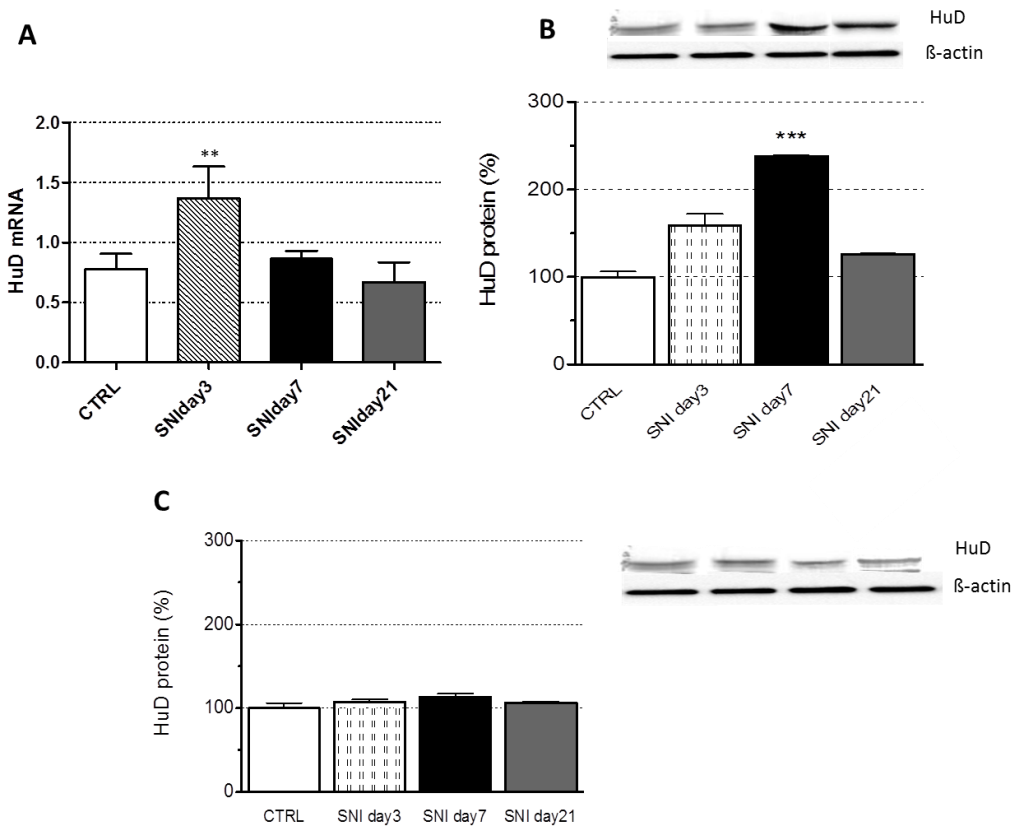
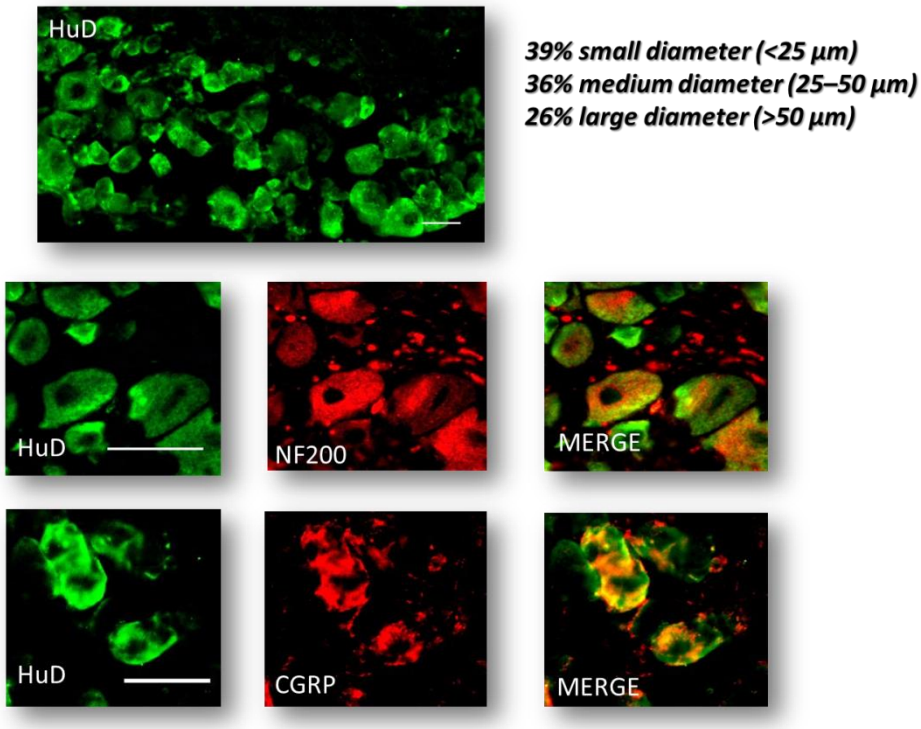


Fig.1 HuD mRNA and protein expression following spared nerve injury. (A) qPCR analysis of RNA levels of HuD in the mouse DRG revealed increased at 3 days. (B) Protein expression increased on day 7 post-injury. (C) No modulation was detected in the mouse spinal cord. **P < 0.01 and ***P < 0.001 in comparison with the non-injured contralateral side.

Fig.2 Characterization of HuD neuronal phenotype in dorsal root ganglia of SNI-mice

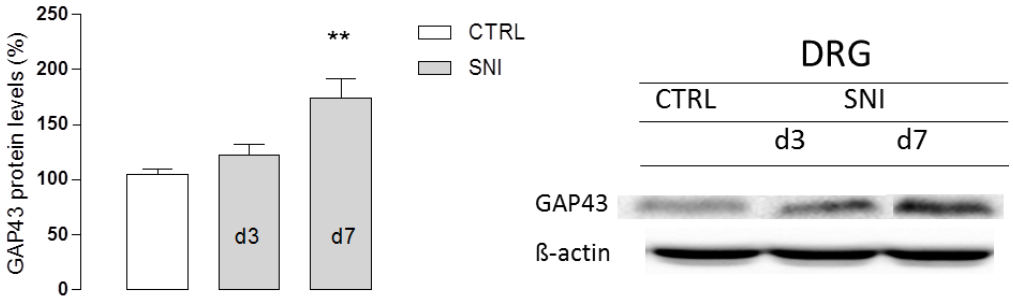
Immunofluorescence experiments revealed that HuD was expressed on the soma of sensory neurons, with more intense staining in small and medium diameters cells. These data are validated with a double staining with NF200 and CGRP, respectively marker of medium and small neurons.

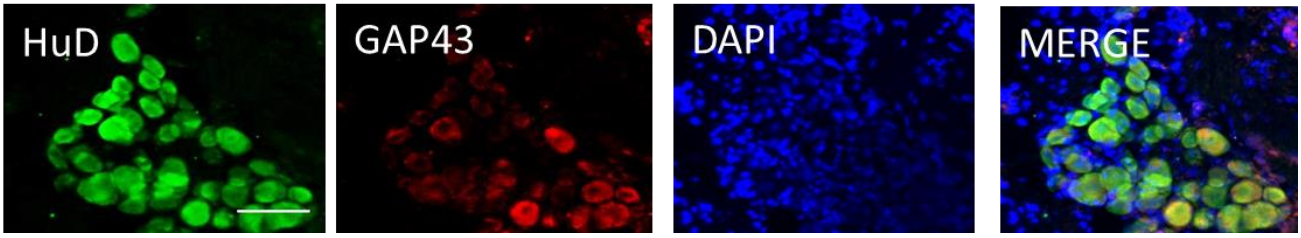


Neuronal phenotype expression of HuD in dorsal root ganglia of spared nerve injury-mice. Scale bar=100 µm

Fig.3 Increasing expression of GAP43 and HuD in DRG regenerating neurons

With regard to the role of HuD, among the numerous processes in which it is involved, this protein accelerates regeneration. Given the known molecular interactions between the GAP43 mRNA and HuD protein, in this study we sought to confirm the role of this RNA-binding protein in regeneration processes after nerve injury. The spared nerve injury increased the expression of GAP43 on day 7 post-injury in coincidence with HuD up-regulation. A colocalization of GAP43 and HuD proteins was found in the soma of regenerating DRG neurons at 7 days.

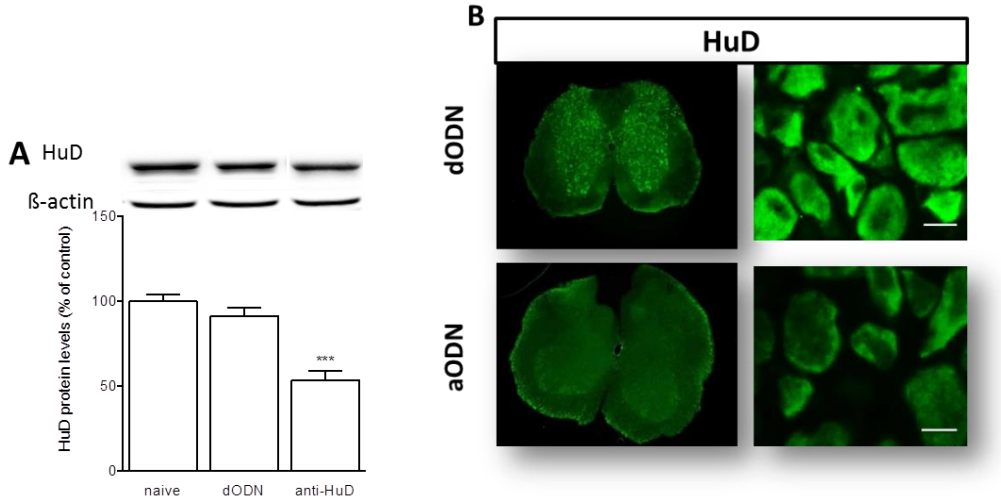




Increased expression of GAP-43 following spared nerve injury on day 7. HuD and GAP-43 protein colocalization in DRG at 7 days post-injury. **P < 0.001 compared to control group. Scale bar=100 μm.

Fig.4 Effect of specific antisense oligonucleotide (aODN) on HuD levels

The sequence-specific knockdown of the HuD protein produced by the aODN treatment was demonstrated by the drastic reduction of HuD levels in the spinal cord whole cell lysate on day 7 (Fig. 4A). Selective overexpression of HuD was confirmed by acute HuD silencing, obtained through the repeated administration of an aODN anti-HuD and compared with the lack of effect of a dODN of the same length and chemical structure. Immunofluorescence images showed the HuD signal in the dODN control group, while following aODN treatment (Fig. 4B) the HuD signal was homogeneously knocked down over the whole spinal cord and DRG sections.



The administration of a specific aODN anti-HuD drastically decreased the HuD protein content in spinal cord lysates. A dODN was used as control ODN treatment. ***P < 0.001 compared with control group. Fluorescence microscopy images showed the reduced expression of HuD within spinal cord and DRG sections following anti-HuD treatment. Scale bar=100 μm.

Fig.5 Role of HuD protein in injury-induced pain hypersensitivity

HuD protein silencing was used to elucidate its role in neuropathic pain induced by spared nerve injury (SNI). Spared nerve injury resulted in a significant decrease in mechanical and thermal

threshold compared to control-mice (Fig. 4A, B). HuD knockdown animals developed less thermal hyperalgesia and mechanical allodynia, without any modulatory effect on pain threshold in the contralateral side (Fig. 4C, D).

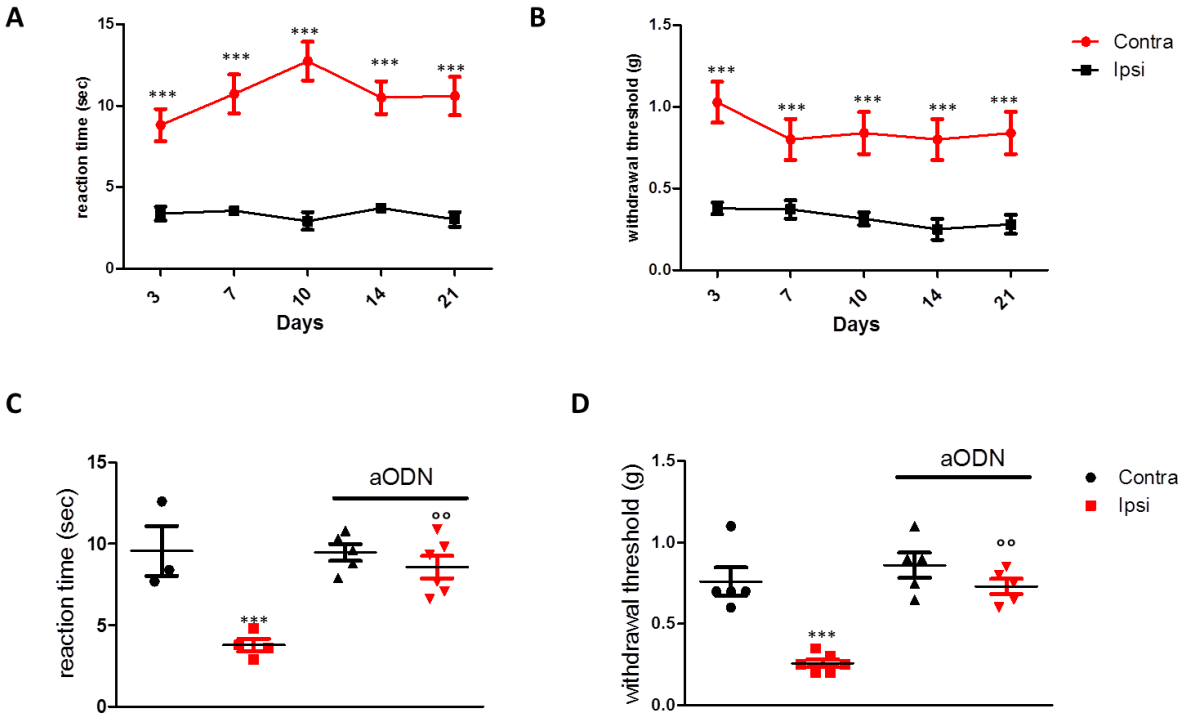


Fig. 5 Prevention by HuD silencing of spared nerve injury-induced pain hypersensitivity. (A, B) Time course of thermal hyperalgesia and mechanical allodynia following spared nerve injury. Treatment with an anti-HuD aODN (1nmol i.t.) prevented thermal hyperalgesia (C) on days 7 post injury and mechanical allodynia (D) without no effect on pain threshold in the non-injured side.***P < 0.001 compared with non-injured side (contra); °°P < 0.01 compared with injured side (ipsi).

Discussion

Within the pain-sensing neurons or nociceptors cellular mechanisms responsible to increase pain hypersensitivity are likely to involve changes in post-trascriptional mechanisms. These post-trascriptional mechanisms from splicing to translation, responsible to regulate protein synthesis are under the control of several RNA-binding proteins (RBPs). Among all RBPs expressed in neurons the best characterized is the neuron specific HuD protein. In this study we discovered that HuD protein is involved not only in regulation of regenerative processes, as already established, but also in the regulation of pain hypersensitivity in a model of spared nerve injury, a useful animal model of neuropathic pain. In particular we found that HuD is up-regulated in dorsal root ganglia of spared nerve injury mice, with a selective expression in small and medium neurons. At the same time point, the levels of GAP43 in the ipsilateral DRG increased relative to the contralateral DRG. Not only were the temporal patterns of expression of HuD protein and GAP43 protein similar, but also they were found to colocalize in the cytoplasm of DRG regenerating neurons. Up-regulation of HuD is also related to the role of this RBP in the regulation of pain threshold, since HuD

knockdown animals developed less evoked pain such as thermal hyperalgesia and mechanical allodynia.
In the second phase we will produce a chronic inhibition of neuronal HuD gene expression through a adeno-associated viral vector (AAV9) that shows neural tropism. To understand its neurobiological role in the peripheral neuropathy we will establish the downstream targets involved in the SNI-induced painful phenotype. Finally, on basis of validated relationship between HuD and mTOR, we will investigate the molecular interaction between mTOR and HuD and if the mTOR expression is related to an altered expression or inadequate mRNA stabilizing properties of HuD after nerve injury.

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