The RNA-binding protein HuD promotes spinal GAP43 overexpression in antiretroviral-induced neuropathy

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ABSTRACT

Nucleoside reverse transcriptase inhibitors (NRTIs) are known to produce painful neuropathies and to enhance states of pain hypersensitivity produced by HIV-1 infection in patients with AIDS leading to discontinuation of antiretroviral therapy, thus limiting viral suppression strategies. The mechanisms by which NRTIs contribute to the development of neuropathic pain are not known. In the current study, we tested the hypothesis that HuD, an RNA binding protein known to be an essential promoter of neuronal differentiation and survival, might be involved in the response to NRTI-induced neuropathy. Antiretroviral neuropathy was induced by single intraperitoneal administration of 2′,3′-dideoxycytidine (ddC) in mice. HuD was physiologically expressed in the cytoplasm of the soma and in axons of neurons within DRG and spinal cord and was considerably overexpressed following ddC treatment. ddC up-regulated spinal GAP43 protein, a marker of neuroregeneration, and this increase was counteracted by HuD silencing. GAP43 and HuD colocalize in DRG and spinal dorsal horn (SDH) axons and administration of an anti-GAP43 antibody aggravated the ddC-induced axonal damage. The administration of a protein kinase C (PKC) inhibitor or the PKCy silencing prevented both HuD and GAP43 increased expression. Conversely, treatment with the PKC activator PDBu potentiated HuD and GAP43 overexpression, demonstrating the presence of a spinal PKC-dependent HuD–GAP43 pathway activated by ddC. These results indicated that HuD recruitment and GAP43 protein increase are mechanistically linked events involved in the response to antiretroviral-induced neurodegenerative processes.

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Introduction

Human immunodeficiency virus (HIV) is most commonly associated with a predominantly sensory polyneuropathy due to viral infection per se or due to a toxic neuropathy associated with antiretroviral treatment (Kamerman et al., 2012; Moore et al., 2000; Schifitto et al., 2002; Simpson et al., 2006). Although differing in etiopathogenesis, these disorders are clinically and physiologically similar, making them difficult to distinguish in individual patients (Dalakas, 2001; Kokozis et al., 2013; Simpson, 2002). Together they are designated HIV-associated sensory neuropathy (HIV-SN),

Abbreviations: ODN, antisense oligonucleotide; ART, antiretroviral therapy; ATF3, activating transcription factor 3; ddC, 2′,3′-dideoxycytidine; dODN, degenerate oligonucleotide; DRG, dorsal root ganglia; ELAV protein, embryonic lethal abnormal vision protein; GAP43, growth-associated protein; HIV-SN, HIV-associated sensory neuropathy; i.p., intraperitoneal; i.t., intrathecal; NRTI, nucleoside reverse transcriptase inhibitors; PKC, protein kinase C; SDH, spinal dorsal horn.

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Patients with AIDS have benefited greatly from the introduction of antiretroviral therapy (ART) (Sacktor, 2002), but both the incidence and prevalence of HIV-SN have risen. Since the introduction in most recent years of combined ART, the prevalence of HIV-SN has continued to rise (Ellis et al., 2010). The drug-induced peripheral neuropathy resulting from ART is most closely associated with the dideoxynucleoside family of nucleoside reverse transcriptase inhibitors (NRTIs), including stavudine (d4T), didanosine (ddI), and zalcitabine (ddC) (Dalakas, 2001; Ellis et al., 2010; Kamerman et al., 2012; Moore et al., 2000). Patients receiving NRTI therapy develop a distal symmetric small fiber “dying back” axonal neuropathy (Dalakas, 2001; Simpson, 2002). Neuropathic pain associated with the use of NRTIs is clinically quite common with a greater prevalence than that reported for other common types of peripheral neuropathy (Luciano et al., 2003; Maritz et al., 2010; Morgello et al., 2004). Unfortunately, analgesics used in other forms of neuropathic pain have proven ineffective for painful HIV-SN (Phillips et al., 2010). The high prevalence of HIV-SN and its negative effect on patients’ quality of life limits antiviral therapeutic options and underscores the need for novel treatment modalities to manage neuropathic pain and to promote neuroregeneration and recovery. A neuroprotective or neuroregenerative strategy would be attractive.
However, no treatments to promote sensory nerve recovery have yet been shown to be effective and safe.

Several studies indicate that nELAV proteins play an important role in neuronal development, differentiation and survival (Pascale et al., 2008). ELAV-like or Hu proteins are a small family of RNA-binding proteins (RBPs). There are four mammalian ELAV-like proteins (HuR, HuB, HuC, and HuD) encoded by separate genes and present in the cell in multiple splice variants. HuB, HuC and HuD, the so-called nELAV proteins, are neuron-specific, while the fourth member, HuR, is ubiquitously expressed (Antic and Keene, 1997; Good, 1995). The biological function of ELAV-like proteins has been identified in their ability to post-transcriptionally promote gene expression up-regulation by cytoplasmic stabilization and/or enhancement of translation of their mRNA targets (Hinman and Lou, 2008). Among the different family members, HuD is recognized as one of the earliest markers of the neuronal lineage as well as being an essential regulator of neuronal differentiation and survival (Deschenes-Furry et al., 2006). The finding that HuD expression increases during learning and memory (Pascale et al., 2004) suggests that this RBP may play an important role in mechanisms of synaptic plasticity in the adult CNS. Recently, it has been reported that overexpression of HuD can rescue motor neuron axonal defects observed in spinal muscular atrophy (SMA)-like conditions (Akten et al., 2011; Hubers et al., 2011). However, the function of this protein in mature neurons is not well understood. In order to elucidate the cellular mechanism of NRTI neuropathy and to investigate into a neuroregenerative strategy for HIV-SN, we sought to investigate the role of HuD in the antiretroviral toxic neuropathy.

We also aimed to elucidate the HuD-mediated signalling pathway. To this purpose we focused on GAP43, a neuronal gene whose mRNA is a target of HuD (Anderson et al., 2001; Moharik et al., 2000), and, as an upstream modulator of HuD, on protein kinase C (PKC). PKCγ activation is generally associated with nociceptive behavior in neuropathic pain conditions (Malmberg et al., 1997; Miletic et al., 2000; Velazquez et al., 2007) and PKC inhibition prevents trauma- and chemotherapy-induced hyperalgesia (Nocini et al., 2009). PKC isoforms are also implicated in the promotion of axonal regeneration, based on the increase in their expression in regenerating neurites and axons (Kawano et al., 1997; Okajima et al., 1995), whereas inhibition of PKC has been found to inhibit the regenerative axonal growth (Campenot et al., 1991; Wiklund and Ekstrom, 1999).

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 behavioral 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 November 24, 1986 (86/609/EEC). All studies involving animals are in accordance with the ARRIVE guidelines for experiments involving animals (McGrath et al., 2010). A total of 120 mice were used in these experiments.

Behavioral testing for mechanical allodynia

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 cm H, 3.4 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. Noxious 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 dDC administration. PWI was quantified by an observer blinded to the treatment.

Preparation of whole cell lysates, membrane and cytosol fractions

The lumbar spinal cord and DRG were removed 1, 3 and 7 days after dDC administration. Samples were homogenized in an homogenization buffer containing 25 mM Tris–HCl pH = 7.5, 25 mM NaCl, 5 mM EGTA, 2.5 mM EDTA, 2 mM NaPP, 1 mM Na3VO4, 1 mM PMSF, 20 μg/ml leupeptin, 50 μg/ml aprotinin, and 0.1% SDS. The homogenate was centrifuged at 9000 × g for 15 min at 4 °C, and the low speed pellet was discarded. The supernatant (whole cell lysate) was centrifuged at 100,000 × g for 60 min at 4 °C. The resulting supernatant was the cytosol fraction, and the pellet was re-suspended in the homogenizing buffer. The homogenate was kept at 4 °C for 60 min with occasional stirring and then centrifuged at 100,000 × g for 60 min at 4 °C. The resultant supernatant was used as membrane fraction. Protein concentration was quantified using Bradford’s method (protein assay kit, Bio-Rad Laboratories, Milan, Italy).

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 washes, blots were incubated overnight at 4 °C with specific antibodies against PKCγ phosphorylated on Thr514 (pPKCγ, 1:1000) (Cell Signalling, MA, USA), PKCγ (1:1000), HuD (1:1000), GAP43 (1:1000), ATF3 (1:1000), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (1:5000) (Santa Cruz Biotechnology Inc, CA, USA). After being washed with PBS containing 0.1% Tween, the nitrocellulose membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antisera (1:10000) 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 (Pierce, Milan, Italy). Exposure 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 housekeeping protein GAPDH at each time point. Measurements in control samples were assigned a relative value of 100%.

Immunofluorescence

Animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). L4–L5 DRGs were removed and postfixed in the same fixative for 2 h prior to paraffin embedding. Sections were cut on a vibratome at 15-micrometer sections. Lumbar spinal cords were removed, postfixed in 4% paraformaldehyde (2 h at 4 °C) and transferred to 20% sucrose (in 0.1 M phosphate buffer) for 2 days at 4 °C. Serial transverse sections of lumbar spinal cord were cut at a thickness of 20 μm. All sections were mounted onto Superfrost Plus microscope slides.

Sections were subjected to antigen retrieval in Na-citrate buffer (10 mM, pH 6) for 20 min at 95 °C. After preincubation in 5 mg/ml BSA/0.3% Triton-X-100/PBS, sections were incubated overnight at 4 °C.
with primary antibody at optimized working dilution. HuD and PKCγ antibodies were detected by Alexa 488-conjugated rabbit secondary antibody (1:200; Invitrogen, Carlsbad, CA). Neurofilament H (1:50) (Santa Cruz Biotechnology Inc, CA, USA), GAP43 (1:50) and NeuN (1:200) antibodies were detected by Alexa 468-conjugated mouse secondary antibody (1:200; Invitrogen, Carlsbad, CA). Sections were coverslipped using Vectorshield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). A Leica DF 350 FX microscope and a Leica SP2-AOBS confocal microscope (Leica Microsystems) with appropriate excitation and emission filters for each fluorophore were used to acquire representative images. Images were acquired with ×10 to ×60 objectives using a digital camera.

To quantitively PKCγ-IR in the spinal cord, five to eight sections from the lumbar spinal cord segments were randomly selected. An image in a square on the medial two-thirds of the superficial dorsal horn (laminas I–III), as described previously by Molander et al. (1984), was captured under ×20 objective, and then an appropriate threshold was set such that only specific PKCγ-IR was accurately represented and light nonspecific background labelling was not detected. The threshold was the same for all images. The density limited to threshold in the outlined area was measured for each section. The relative PKCγ-IR density was shown as the ratio of the density of the treated groups to the control group. A similar approach has been used previously (Martin et al., 1999).

Histological analysis

Histological analyses were performed as described by Bhasin et al. (2007). Mice were anesthetized and then perfused with 4% paraformaldehyde. Sciatic nerve was removed on day 7 post-injection and frozen for sectioning. Transverse sections (5 μm) were incubated overnight at 56 °C in 0.1% Liquol Fast Blue (Sigma, St. Louis, MO) in 95% ethanol and glacial acetic acid. The slides were then rinsed in 95% ethanol and distilled water and differentiated successively in 0.1% lithium carbonate and 70% ethanol. After dehydration and xylene treatment, the slides were coverslipped using Permount. LFB-positive area in which the density significantly exceeded the threshold of each background was calculated as the percentage cross-sectional area of residual tissue. The total myelinated area, the diameter of the nerve and the number of myelinated axons were obtained using the NIH Image J 1.36b imaging software (NIH, Bethesda, MD, USA) and applied to all experimental groups. The total pixel intensity was determined, and the data are expressed as luxol fast blue positive area (%).

Drug administration

ddc (Sigma, Milan, Italy) solution was freshly prepared in saline (0.9% NaCl) on the day of the experiment. ddc- and vehicle-treated groups were given a one-time intraperitoneal (i.p.) injection of ddc (25 mg/kg) or saline, respectively. Experiments were performed on day 1 (absence of neuropathic pain), 3 and 7 (presence of neuropathic pain and neuronal damage).

The PKC blocker calphostin C (0.2 μg per mouse) and the PKC activator phorbol-12,13-dibutyrate (PDBu; 40 pmol per mouse) (Calbiochem, Milan, Italy) were dissolved in 0.5% DMSO and administered intrathecally (i.t.), as previously described (De la Calle and Paino, 2002). Time-course experiments performed in our laboratory showed that the calphostin C and PDBu effect peaked 1–2 h after administration (Galeotti et al., 2003). Animals receiving PKC modulators were, then, divided in the following groups:

1) Calphostin C or PDBu administered on day 1, 2 and 3 and protein expression detected on day 3, 1 h after the last injection;

2) Calphostin C administered on day 3, 4, 5 and 7 and protein expression detected on day 7, 1 h after the last injection;

3) PDBu or calphostin C single administration 1 h before ddc injection and behavioral tests performed on day 3;

4) Calphostin C administered for 3 consecutive days on day 1, 2, 3 or 8, 9, 10, and the behavioral test performed 1 h after the last injection.

Antisense oligonucleotide administration

Phosphodiester oligonucleotides (ODNs) protected from terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were obtained from Tib Molbiol (Genoa, Italy). The ODN against PKCγ was the following: 5'-A-G'-GAATCCGGGTCACAT'A-3' where the asterisks indicate the phosphorothioate phosphate groups, and it was previously characterized by in vitro and in vivo experiments in our laboratory (Galeotti et al., 2006). The ODN against HuD was the following: 5'-21-G'-TCTGGAGCCTAC'T'T'-3' where the asterisks indicate the phosphorothioate 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 the HuD protein knockdown, mice received a single i.t. injection every 24 h on day 1, 2 and 3 for a total of 3 injections. The spinal cord was removed on day 3, 12 h after the last injection, and on day 7.

Statistical analysis

Western blotting experimental results are given as the mean ± s.e.m. of band intensities obtained from 6 to 8 independent experiments; three mice per group were used. Behavioral results are given as mean ± s.e.m.; eight mice per group were used. One-way and two-way analysis of variance, followed, respectively, by Tukey and Bonferroni post hoc test, were used for statistical analysis.

Results

Increased expression of HuD in ddc-induced neuropathy

Following antiretroviral treatment, a progressive increase of HuD protein levels within the spinal cord and DRG was observed (Fig. 1A).

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 throughout the entire grey matter area in the dODN control group (Fig. 1B), while following aODN treatment the HuD signal was homogeneously knocked down over the whole spinal cord section (Fig. 1C). 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 (Fig. 1D). HuD silencing also prevented the ddc-induced HuD up-regulation in the whole cell lysate (Fig. 1D) and cytosol fraction (Fig. 1E) 3 and 7 days after antiretroviral administration.

Tissue and cellular localization of HuD

HuD is a cytosolic protein also expressed in axons. To determine the cellular localization of HuD in our experimental conditions, double labelling immunostaining with classical markers of subpopulations was carried out in the mouse spinal cord sections. We found that in the SDH HuD (Fig. 1F) colocalized with NeuN (Fig. 1H), a neuronal marker (Fig. 1G), confirming the neuronal localization of spinal HuD. Investigating into HuD subcellular localization in the spinal cord, double labelling immunostaining of HuD with DAPI showed the presence of HuD in the cytoplasm and its absence in the nucleus (Figs. 1L–K). Cytosolic distribution of HuD was also confirmed in the DRG (Figs. 1L–Q). Confoocal fluorescence microscopy images illustrated the co-localization of
HuD with neurofilament H, an axonal marker, revealing the axonal localization of HuD within SDH (Figs. 1R-T).

PKCy as upstream modulator of HuD

To investigate cellular mechanisms that promote HuD upregulation, the protein expression and phosphorylation of PKCy, a neuronal PKC isoform, were examined. ddC produced a long lasting increased expression and phosphorylation of spinal PKCy (Figs. 2A and C). A higher increase in the phosphorylation of PKCy was observed in the membrane fraction whereas in the cytosol fraction the levels of pPKCy were unmodified at all time points, indicating that ddC promoted the translocation of the PKCy isozyme from the cytosol to the membrane (Fig. 2A).

The PKC blocker calphostin C (CC) and PKCy silencing prevented the ddC-induced pPKCy increase. Furthermore, the PKCy silencing produced a knockdown of the total and phosphorylated enzyme in control mice (Figs. 2A and B).

HuD knockdown altered neither PKCy–pPKCy basal levels nor the ddC-induced PKCy–pPKCy overexpression, confirming PKC as upstream modulator of HuD (Fig. 2C). Immunofluorescence images further confirmed this hypothesis. Positive immunofluorescence for PKCy was observed in interneurons in laminae II and III and in the dorsal funiculus in untreated control mice. CC did not modify the PKCy immunostaining, but counteracted ddC-induced increase of PKCy immunolabelling (Figs. 2D–G; high magnification H–K). No unspecific labelling following ddC or CC treatment was observed.
CC prevented HuD increase whereas the PKC activator PDBu significantly increased the HuD upregulation further demonstrating the PKC-dependent HuD activation (Fig. 3A). Moreover, the PKC-γ knockdown reduced HuD protein levels in untreated control mice and prevented the ddC-induced overexpression. (C) The antiretroviral administration increased the spinal PKC-γ and pPKC-γ content that remained unmodified after HuD silencing (anti-HuD). In the figure the effect observed on day 3 post-injection as representative is reported. Fluorescence microscopy images show the expression of PKC-γ throughout the SDH in untreated control animals (CTRL; D) and following treatment with CC (E), ddC (F), ddC + CC (G). H, I, J and K are higher magnification images of boxes in D, E, F and G, respectively. PKC-γ immunoreactivity is apparent in lamina II and III of SDH and in the ventral portion of the dorsal funiculus. Scale bars = 100 μm.

**Fig. 2.** ddC increased the expression and phosphorylation of protein kinase C-γ (PKC-γ) as upstream modulator of HuD. (A) ddC increased the expression of pPKC-γ within spinal cord 3 and 7 days after administration. Subcellular fractioning showed a similar upregulating effect induced by ddC on pPKC-γ levels in the membrane fraction (mem). No modification of pPKC-γ contents was detected on the cytosol fraction (cyt). The pPKC-γ upregulation was reversed by the PKC blocker calphostin C (CC). (B) The PKC-γ protein knockdown (anti-PKC-γ) reduced tot PKC-γ and pPKC-γ in control mice and prevented the ddC-induced overexpression. (C) The antiretroviral administration increased the spinal PKC-γ and pPKC-γ content that remained unmodified after HuD silencing (anti-HuD). In the figure the effect observed on day 3 post-injection as representative is reported. Fluorescence microscopy images show the expression of PKC-γ throughout the SDH in untreated control animals (CTRL; D) and following treatment with CC (E), ddC (F), ddC + CC (G). H, I, J and K are higher magnification images of boxes in D, E, F and G, respectively. PKC-γ immunoreactivity is apparent in lamina II and III of SDH and in the ventral portion of the dorsal funiculus. Scale bars = 100 μm.

With regard to the role of HuD and PKC, among the numerous processes in which they are involved, both proteins accelerate regeneration. The presence of a PKC-γ-dependent HuD upregulation encouraged us to clarify the existence of regeneration responses induced by ddC treatment. We, therefore, investigated the expression and localization of GAP43, a marker of neuronal regeneration. The antiretroviral drug
drastically increased the expression of spinal GAP43, in the whole cell lysate and cytosol fraction, on day 3 and 7 post-injection with a time-course consistent with HuD up-regulation (Figs. 4A and B). GAP43 expression appeared to be physiologically related to the presence of HuD, since knockdown of HuD reduced GAP43 content in control mice. HuD silencing also prevented the ddC-induced GAP43 overexpression (Figs. 4A and B). Simultaneously to the GAP43 increase, we detected a reduction of activating transcription factor 3 (ATF3), a marker of neuronal degeneration, that was prevented by HuD silencing (Fig. 4C).

Sciatic nerve sections showed a ddC-induced neuronal damage characterized by increased myelin content (Figs. 4D and H). Intrathecal administration of an antibody anti-GAP43 accentuated the neuronal damage by inducing a strong reduction of myelin content (Figs. 4D and I) and by increasing the axonal diameter (Figs. 4E and I). No variation of the number of fibers was detected (Figs. 4F-I).

PKC-dependent activation of the HuD/GAP43 pathway

To finally identify the cellular pathway responsible for the up-regulation of GAP43 following ddC treatment, we checked the expression of this neuroregeneration marker following PKC inactivation. PKCy protein knockdown reduced GAP43 protein levels in control mice and prevented ddC-induced GAP43 overexpression (Fig. 4J). Similarly, treatment with CC completely prevented the antiretroviral-induced up-regulation of GAP43 on day 3 and 7 post-injection (Figs. 4J and K). Administration of the PKC activator PDBu significantly increased the GAP43 up-regulation (Fig. 4K).

PKCy is a PKC isoform highly involved in pain processes. We, therefore, evaluated if the observed PKC activation, besides being involved in neuroregeneration processes, was also related to the ddC-induced nociceptive behavior. ddC produced a long lasting mechanical allodynia. The threshold to the mechanical stimulus was reduced as early as day 3.
post-injection and remained unchanged up to day 14 post-injection (Fig. 5A). Post-ddC administration of CC was devoid of any antiallodynic activity in mice exposed to the antiretroviral (Fig. 5B). To determine if a modulation of the PKC activity might have any preventive effect on ddC-induced mechanical allodynia, animals were pre-treated with a single administration of the PKC activator PDBu or of the PKC blocker
GAP43 immunostaining was present in all regions of the grey matter. Granular material in the soma of DRG neurons (Fig. 6A). Similarly, GAP43 immunostaining was distributed throughout the neuropil of the entire grey matter. The intensity of the neuropil immunoreactivity was almost uniform within the SDH (Figs. 6E, H, and K). Subcellular localization studies showed an axonal and cytoplasmic distribution of GAP43 immunostaining (Fig. 6K). Co-localization of HuD and GAP43 proteins was observed in primary afferent axons, in the DRG axons projecting to the substantia gelatinosa and some colocalization of HuD with GAP43 was also observed within axons of the substantia gelatinosa neurons, indicating the expression of HuD in regenerating axons (Figs. 6F and I). Confocal fluorescence microscopy images illustrated a co-localization of HuD and GAP43 in the soma and axons within the substantia gelatinosa (Figs. 6J–L). The same pattern of HuD and GAP43 expression and colocalization was also detected in untreated control mice (Figs. 6M, N, and O), supporting the hypothesis of a physiological GAP43 mRNA stabilizing activity exerted by HuD in the mouse spinal cord.

**Discussion**

The most consistent associations with HIV-SN risk are exposure to potentially neurotoxic NRTIs stavudine (d4T), zalcitabine (ddC) and didanosine (ddI) (Ances et al., 2009; Maritz et al., 2010). However, elucidation of the specific neuropathological processes that ultimately cause nerve fiber dysfunction is confounded in HIV-infected individuals by concomitant viral-induced nerve damage. Therefore, delineation of the mechanisms that underlie the drug-induced component of the neuropathy has required the development of in vitro and in vivo models of pure NRTI-induced neurotoxicity. However, animal models of pure NRTI-induced neuropathy have yielded mixed results, with only ddC producing reliable models (Kamerman et al., 2012). Despite the drug no longer being used clinically (WHO et al., 2010), ddC-based animal models have formed the basis for investigations into the mechanisms of NRTI-induced neuropathology and neuropathic pain-like behaviors (Kamerman et al., 2012).

We illustrated the presence of a HuD–GAP43 mediated pathway within DRG and SDH that results activated in the early phases of the neurodegenerative process induced by ddC. This phenomenon is shown by a rise in the expression of the GAP43 protein, the most widely used marker of nerve regeneration (Griffin et al., 2010). Several studies showed that a single injection of ddC resulted in structural damage, small sensory fiber axonal neuropathy (Dalakas, 2001; Venhoff et al., 2010; Wallace et al., 2007). Neuropathological changes in peripheral nerves, such as redundant myelin loop formation and diminished axonal cytoplasm, have been detected as early as the day 3 post-injection (Bhango et al., 2007). These neuronal alterations have been observed by us and confirmed also at day 7 post-injection. The sequestration of GAP43 through intrathecal administration of a specific antibody further aggravated the ddC-induced neuronal damage. The GAP43 overexpression appears functional, making this spinal pathway a cellular event involved in the response to the ddC-induced neuropathy.

The increase of GAP43 protein appeared simultaneously to an upregulation of the RNA binding protein HuD, a member of the neuronal ELAV proteins. Among the neuronal genes whose mRNA is a target of the nELAV proteins, GAP43 is the most well-characterized. Expression of the GAP43 gene is regulated at both the transcriptional and post-transcriptional levels, with the latter involving changes in the stability of its mRNA leading to an increased mRNA half-life. The GAP43 mRNA has a highly conserved 3′ untranslated region (3′UTR) with an AU-rich element (ARE), conferring a short half-life to the GAP43 transcript. However, this site is recognized by HuD and other sequence-specific RBPs (Perrone-Bizzozero and Bolognani, 2002; Tsai et al., 1997) whose binding can stabilize the GAP43 mRNA. The stabilization of GAP43 mRNA by HuD has been demonstrated in cell culture, in vitro and in vivo. In PC12 cells, primary cortical neurons, and retinoic acid-

**Colocalization of HuD and GAP43 within mouse SDH axons and DRG**

To clarify the cellular localization of the ddC-induced neuroregeneration process, the co-expression of HuD and GAP43 was investigated. In ddC-treated mice HuD protein was found to localize to granular material in the soma of DRG neurons (Fig. 6A). Similarly, GAP43 protein appeared to localize in granules of the cytosol of regenerating DRG neurons (Fig. 6B) and showed a high degree of co-expression with HuD (Fig. 6C). Following ddC administration, HuD immunostaining was distributed throughout the lumbar spinal cord grey matter (Fig. 6D). Subcellular localization studies showed an axonal and cytoplasmic distribution of HuD immunostaining (Figs. 6G and J). GAP43 immunostaining was present in all regions of the grey matter.

**Fig. 5.** Effect of PKC activity modulation on ddC-induced mechanical allodynia. (A) ddC induced a prolonged mechanical allodynia starting from 3 days after injection. ***p < 0.001 compared with control group. (B) Lack of effect of post-ddC repeated administration of calphostin C on ddC-induced mechanical allodynia. # ***p < 0.001 compared with before treatment value. (C) Pretreatment with the PKC blocker calphostin C (CC) or the PKC activator PDBu 1 h before ddC injection did not exert any preventive activity on mechanical allodynia. ***p < 0.001 vs. corresponding control value.
differentiated embryonic stem cells transfected with HuD, GAP43 mRNA stability is increased resulting in enhanced gene expression and neurite outgrowth (Anderson et al., 2001; Mobarak et al., 2000). The function of HuD on GAP43 expression was also demonstrated in neurons in vivo. GAP43 and HuD expression overlaps in many sites of the mature brain, including the hippocampus (Pascale et al., 2004; Perrone-Bizzozero and Bolognani, 2002) and the overexpression of HuD in forebrain neurons results in the stabilization of GAP43 mRNA (Bolognani et al., 2006). A study using viral vectors to express HuD in regenerating superior cervical ganglion neurons (Deschênes-Furry et al., 2007) indicated that HuD stabilizes the GAP43 mRNA during nerve regeneration. A HuD-dependent GAP-43 mRNA increase has been shown in the DRG and axons during PNS nerve regeneration in a peripheral neuropathy induced by nerve injury (Anderson et al., 2003; Yoo et al., 2013).

We observed a co-localization of HuD and GAP43 in the DRG and SDH cell bodies, distal axons and growth cones after ddC treatment, and the HuD gene expression knockdown completely prevented the

![Fig. 6. Co-localization of HuD and GAP43 proteins in the SDH and DRG following ddC treatment. Tissue sections harvested 7 days after ddC administration were double-immunostained for HuD (green; A, D, G, J, and M) and the well-established regeneration-associated marker GAP43 (red; B, E, H, K, and N) to determine co-localization (merged images; C, F, I, L, and O). In the DRG HuD protein was localized to fine granules throughout the cytoplasm (A). GAP43 immunostaining is apparent as granular staining in both large and small diameter cells following ddC administration (B). HuD immunostaining in the same section reveals a high degree of co-expression with the regeneration-associated marker (HuD and GAP43 merged, C). HuD immunostaining was distributed throughout the SDH (D) and showed a cytoplasmic and axonal distribution (D and G). GAP43 immunostaining is apparent in axons and throughout the neuropil (E and H). Merged images reveal a high degree of coexpression of HuD and GAP43 in regenerating axons (F and I). G, H, and I are higher magnification images of boxes in D, E, and F, respectively. Scale bars = 50 μm. Confocal fluorescence representative images of the substantia gelatinosa showed the cytoplasmic and axonal HuD immunostaining, the neuropilar GAP43 immunostaining and the co-localization of HuD and GAP43 within axons in ddC-treated mice (J, K, and L) and in control untreated mice (M, N, and O). Scale bars = 10 μm.](https://example.com/fig6.png)
overexpression of GAP43 induced by antiretroviral administration. These observations further support the hypothesis of a role of HuD in the response to peripheral nerve degeneration, indicating the need of the gene expression promoting activity of HuD to increase the GAP43 content in animals exposed to antiretroviral treatment. We also showed a co-localization of HuD and GAP43 in cell bodies and axons of untreated control mice, and reduced GAP43 spinal levels in control animals after HuD silencing. These data might also indicate the presence of a HuD–GAP43 spinal pathway physiologically active in the absence of a neuronal damage.

The present work also demonstrated the existence of a pathway involving PKCy for HuD recruitment and activity. A robust upregulation and increased phosphorylation of PKCy, a neuronal PKC isoform, have been detected in the spinal cord of mice following a single ddC administration. The translocation of PKC enzymes from cytosol to the synaptic membrane is thought to be necessary for their activation. As confirmation of a specific PKC activation, we observed a robust increase of the PKCy phosphorylation in the membrane fraction, whereas a lack of increase in any of the phosphorylations in the cytosol was detected. The time-course of PKCy hyperphosphorylation was consistent with HuD and GAP43 overexpression, and immunofluorescence experiments showed that PKCy, HuD and GAP43 are all localized in the superficial laminae of the SDH (substantia gelatinosa). Pharmacological PKC blockade and PKCy silencing prevented the PKCy hyperphosphorylation and counteracted the increase of HuD and GAP43 in ddC-treated mice. PKCy silencing also decreased the expression of HuD and GAP43 in the absence of a neuronal damage (untreated control mice). In PC12 pheochromocytoma cells exposed to nerve growth factor (NGF), HuD has been proposed as an essential factor for PKC-dependent neurite outgrowth through the control of GAP43 mRNA stability and GAP43 expression (Mobarak et al., 2000). HuD phosphorylation by another PKC isoform, PKCα, resulted in redistribution of the proteins and increased stabilization of GAP43 in neuroblastoma cells (Pascale et al., 2005). We have recently observed the PKCγ-mediated cerebral overexpression of HuD and GAP43 in a condition of neurotoxicity (Sanna et al., 2014). We, here, demonstrated the presence of a specific PKCy–HuD–GAP43 pathway in the SDH, which is activated in the response to ddC treatment and that might be involved in a nerve regeneration process.

In painful neuropathies PKCy activation is generally associated with the nociceptive phenotype that is prevented by PKC blockers. However, we observed that a pharmacological blockade of PKCy did not attenuate ddC-induced mechanical allodynia, in agreement with previous results in a rat model (Joseph et al., 2004). Conversely to other types of neuropathic pain, in the ddC model any direct role of PKC in the induction of hyperalgesia can be excluded. We can, hence, assume that the activation of PKCy-mediated pathways induced by the antiretroviral treatment might selectively be involved in a neuroregeneration process without any direct role in pain hypersensitivity. Since analgesics clinically used to treat neuropathic pain have proven ineffective for painful HIV-SN (Phillips et al., 2010), a therapeutic strategy that promotes modulation of neuroregenerative processes might have a clinical relevance by improving the antiretroviral-induced neuropathy and, hopefully, recovering from pain.

Conclusions

Since NRTI introduction, the morbidity and mortality of HIV infection have been markedly reduced (Kameran et al., 2012). However, the drug-induced neuropathy may precipitate abbreviation or discontinuation of antiretroviral therapy thus limiting viral suppression strategies. Ideally, a pathogenesis-based treatment for HIV-SN would allow patients to avoid discontinuation of NRTI therapy with neurotoxic antiretroviral drugs. Present findings highlight the presence of a spinal PKC-mediated pathway activated in the response to ddC neuropathy, involving the GAP43 mRNA stabilizing activity of HuD. We can propose the activation of the PKC–HuD pathway as a therapeutic perspective to promote neuroprotection/neuroregeneration towards antiretroviral painful neuropathy.

Statement of interest

The authors declare none.

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