Experimental Neurology 261 (2014) 343–353

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Regular Article

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

M.D. Sanna [a], A. Quattrone [b], T. Mello [c], C. Ghelardini [a], N. Galeotti [a,*]

[a] Laboratory of Neuropsychopharmacology, Department of Neurosciences, Psychology, Drug Research and Child Health (NEUROFARBA), Section of Pharmacology, University of Florence, Florence, Italy
[b] Laboratory of Translational Genomics, Centre for Integrative Biology, University of Trento, Trento, Italy
[c] Department of Biomedical, Experimental and Clinical Sciences, University of Florence, Florence, Italy

ARTICLE INFO

Article history:
Received 7 February 2014
Revised 10 May 2014
Accepted 16 May 2014
Available online 24 May 2014

Keywords:
Antiretroviral
Neuropathy
HuD
GAP43
Protein kinase C

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 a 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 PKCγ 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.

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; Kokotis et al., 2013; Simpson, 2002). Together they are designated HIV-associated sensory neuropathy (HIV-SN).

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.

Abbreviations: aODN, 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.

* Corresponding author at: Department of NEUROFARBA, Viale G. Pieraccini 6, I-50139 Florence, Italy. Fax: +39 055 4271280.
E-mail address: nicoletta.galeotti@unifi.it (N. Galeotti).

http://dx.doi.org/10.1016/j.expneurol.2014.05.017
0014-4886/

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 (Deschênes-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 deficits 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; Mobarak et al., 2000), and, as 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 (Norcini 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 reported 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. 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 ddC administration. PWT 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, 4 mM PNFF, 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 (1:10,000) 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 (Pierce, Milan, Italy). 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 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