ELSEVIER

Neurobiology of Aging 32 (2011) 2016–2029

NEUROBIOLOGY OF AGING

www.elsevier.com/locate/neuaging

Declining phosphatases underlie aging-related hyperphosphorylation of neurofilaments

Veeranna [a,c], Dun-Sheng Yang [a,c], Ju-Hyun Lee [a,c], K. Yaragudri Vinod [b], Philip Stavrides [a], Niranjana D. Amin [e], Harish C. Pant [e], Ralph A. Nixon [a,c,d,*]

[a] *Center for Dementia Research, Nathan Kline Institute for Psychiatric Research, Orangeburg, NY 10962, United States*
[b] *Division of Analytical Psychopharmacology, Nathan Kline Institute for Psychiatric Research, Orangeburg, NY 10962, United States*
[c] *Department of Psychiatry, New York University School of Medicine, New York, NY 10016, United States*
[d] *Department of Cell Biology, New York University School of Medicine, New York, NY 10016, United States*
[e] *Laboratory of Neurochemistry NINDS, NIH, Bethesda, Maryland, MD 20892, United States*

Received 28 September 2009; received in revised form 24 November 2009; accepted 2 December 2009
Available online 23 December 2009

Abstract

Cytoskeletal protein phosphorylation is frequently altered in neuropathologic states but little is known about changes during normal aging. Here we report that declining protein phosphatase activity, rather than activation of kinases, underlies aging-related neurofilament hyperphosphorylation. Purified PP2A or PP2B dephosphorylated the heavy neurofilament (NFH) subunit or its extensively phorphorylated carboxyl-terminal domain *in vitro*. In cultured primary hippocampal neurons, inhibiting either phosphatase induced NFH phosphorylation without activating known neurofilament kinases. Neurofilament phosphorylation in the mouse CNS, as reflected by levels of the RT-97 phosphoepitope associated with late axon maturation, more than doubled during the 12-month period after NFH expression plateaued at p21. This was accompanied by declines in levels and activity of PP2A but not PP2B, and no rise in activities of neurofilament kinases (Erk1,2, cdk5 and JNK1,2). Inhibiting PP2A in mice *in vivo* restored brain RT-97 to levels seen in young mice. Declining PP2A activity, therefore, can account for rising neurofilament phosphorylation in maturing brain, potentially compounding similar changes associated with adult-onset neurodegenerative diseases.

Keywords: Neurofilament; Phosphorylation; Dephosphorylation; Kinases; Phosphatases; Maturation; Aging; RT-97 epitope; Immunoreactivity

1. Introduction

Neurofilaments (NFs) are the major cytoskeletal components of large myelinated axons and their subunits are among the most highly phosphorylated brain proteins. NFs in the central nervous system (CNS) consist of the four subunits, α-internexin, NFH (High), NFM (Medium), and NFL (Low) (Chin and Liem, 1990; Yuan et al., 2009), each containing a short head domain, an α helical rod domain, and a carboxyl-terminal tail domain that varies in length depending on the subunit. The exceptionally long tail domains of NFM and NFH contain 5 and 52 KS/TP repeats, respectively, which are endogenously phosphorylated by proline directed kinases (Nixon and Sihag, 1991; Pant et al., 2000; Sihag et al., 2007) (Fig. 1).

Phosphorylation causes the tail domains to project perpendicularly from the neurofilament and act as "sidearms" that regulate filament spacing (Nixon and Sihag, 1991) (Elder et al., 1998; Mukhopadhyay et al., 2004; Sihag et al., 2007). These events stabilize a large cross-linked NF network that is a structural framework enabling the marked expansion of axon caliber during maturation required for proper impulse conduction (Gasser and Grundfest, 1939; Nixon and Lewis, 1986; Nixon and Logvinenko, 1986) and the optimal

* Corresponding author at: Nathan Kline Institute for Psychiatric Research, New York University School of Medicine, 140 Old Orangeburg Rd., Orangeburg, NY 10962, United States. Tel.: +1 845 398 5423; fax: +1 845 398 5422.
E-mail address: nixon@nki.rfmh.org (R.A. Nixon).

0197-4580/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.neurobiolaging.2009.12.001

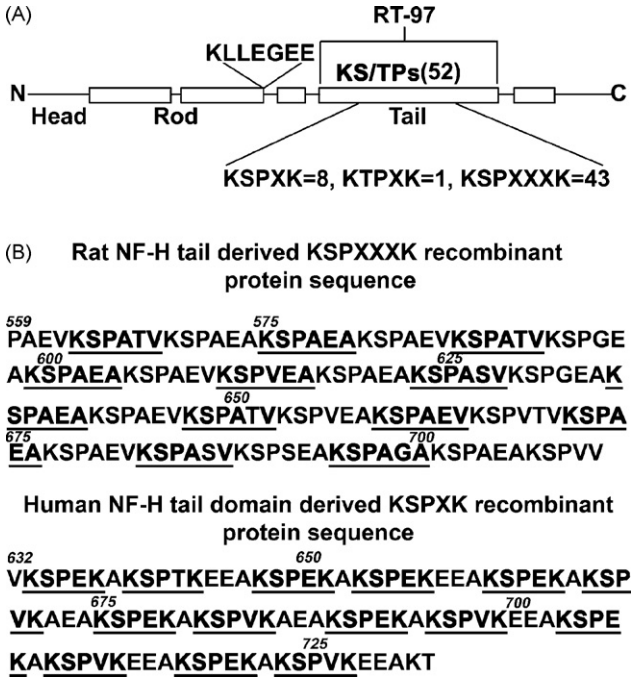

Fig. 1. A cartoon of NF-H shows its polypeptide domains (A) and sequences of the KSPXXXK and KSPXK type of recombinant proteins derived from the rat and human NF-H tail domains respectively (B).

topographical organization of vesicular organelles and receptors within axons and synaptic terminals (Ehlers et al., 1998; Kim et al., 2002; Perrot and Julien, 2009). The appearance of the phosphorylation-dependent epitope RT-97 on NFH and NFM sidearms is an important marker of the establishment of a metabolically stable stationary cytoskeleton and the axon caliber growth initiated after axons establish synaptic connections and acquire myelin. Importantly, increased levels of the RT-97 phosphoepitope on NF proteins and tau, especially in neuronal perikarya, identifies affected neurons in certain neurodegenerative diseases, including Alzheimer's disease (AD), where cytoskeletal protein hyperphosphorylation is believed to contribute to disease pathogenesis (Anderton et al., 1982; Kesavapany et al., 2007; Sternberger et al., 1985; Veeranna et al., 2004).

The RT-97 phosphoepitope has been shown to be regulated by MAPK family members, such as Erks and JNKs, that phosphorylate KSPXK and KSPXXXK motifs along NFH and NFM tail domains (Giasson and Mushynski, 1997; Veeranna et al., 1998) and also by cdk5, which phosphorylates only KSPXK sites (Shetty et al., 1993). Evidence that phosphate groups turn over on NF proteins *in vivo* (Nixon and Lewis, 1986) suggests that phosphatases may also be important in regulating phosphate topography along cytoskeletal polypeptides, as underscored by studies of cytoskeletal proteins in neurodegenerative diseases (Gong et al., 1993, 1995, 2000; Kesavapany et al., 2007; Veeranna et al., 2004). In AD brain, for example, abnormal hyperphosphorylation of NF and tau is accompanied by significantly decreased mRNA expression, protein levels (Vogelsberg-Ragaglia et al., 2001), and methylation (Sontag et al., 2004) of protein phosphatase 2A (PP2A) and lowered protein levels of protein phosphatase 1 (PP1) (Gong et al., 1993, 1995, 2000). These changes are believed to compound effects of additional disease-related activation of certain protein kinases that can phosphorylate NF proteins and tau (Ferrer et al., 2005; Pei et al., 2002; Veeranna et al., 2004; Wang et al., 2001; Webber et al., 2005).

Despite the intense interest in the abnormal phosphorylation of the cytoskeleton in relation to disease pathogenesis, changes in the state of phosphorylation of cytoskeletal proteins during the course of normal brain maturation and aging are not well characterized or understood in terms of underlying molecular mechanisms. In the present study, we investigated these issues in hippocampal neurons and the normal mouse CNS. These studies strongly implicate declining activity of PP2A in aging brain. This decline would be expected to compound similar changes in phosphatase activities and protein hyperphosphorylation in major aging-related neurodegenerative diseases, thereby, providing further understanding of how brain aging may contribute to late-onset neurological disease.

2. Materials and methods

2.1. Mice, antibodies and other reagents

Aged C57BL6 mice (Rodent Resource Facility of the National Institute of Aging, Charles River Laboratories, Wilmington, MA) acclimatized for one week in the Nathan S. Kline Institute for Psychiatric Research (NKI) animal facility. All animal experiments were performed according to "Principles of Animal Care" (NIH, 1985) and approved by the Institutional Animal Care and Use Committee at the NKI. We obtained the following antibodies commercially, monoclonal antibody SMI-33 dephospho-epitope on NFH and NFM (Sternberger Monoclonal Inc., Baltimore, MD); polyclonal antibodies against p35 and cdk5 (Santa Cruz Biotechnology Inc., Santa Cruz, CA); phospho-Erk1,2, phospho-independent Erk1,2, phospho JNK 1,2, JNK 1,2 and antibodies to catalytic subunits of PP2A and PP2Bα (Cell Signaling, Boston, MA) and PP1 assay kit (New England Biolabs Inc, Ipswich, MA). A rabbit polyclonal antibody against NFL was made in our laboratory. The RT-97 monoclonal antibody clone was a kind gift from Brian Anderton (Institute of Psychiatry, London). Anti-mouse and anti-rabbit secondary antibodies conjugated to alkaline phosphatase (Promega, Madison, WI); or horseradish peroxidase conjugated anti-mouse and anti-rabbit secondary antibodies (Jackson Immunochemical Laboratories, Baltimore, MD); or Alexa 488 and Alexa 568 tagged secondary antibodies and all cell culture reagents (Invitrogen, Carlsbad, CA). Recombinant Erk2 and MEK1 proteins were kind gifts from Dr. N.G. Ahn (University of Colorado, Boulder, CO). Additional commercial reagents included microcystin LR (Calbiochem, San Diego, CA); okadaic acid (OA) and cyclosporine A (Biomol,