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Sustained high levels of neuroprotective, high molecular weight, phosphorylated tau in the longest-lived rodent

Miranda E. Orr, Valentina R. Garbarino, Angelica Salinas, Rochelle Buffenstein*

Department of Physiology and The Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

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ABSTRACT

Tau protein is primarily expressed in neuronal axons and modulates microtubule stability. Tau phosphorylation, aggregation, and subcellular mislocalization coincide with neurodegeneration in numerous diseases, including Alzheimer's disease (AD). During AD pathogenesis, tau misprocessing accompanies Aß accumulation; however, AD animal models, despite elevated Aß, fail to develop tauopathy. To assess whether lack of tau pathology is linked to short life span common to most AD models, we examined tau processing in extraordinarily long-lived, mouse-sized naked mole-rats (NMRs; approximately 32 years), which express appreciable levels of Aß throughout life. We found that NMRs, like other mammals, display highest tau phosphorylation during brain development. Although tau phosphorylation decreases with aging, unexpectedly adult NMRs have higher levels than transgenic mice overexpressing mutant human tau. However, in sharp contrast with the somatodendritic accumulation of misprocessed tau in the transgenic mice, NMRs maintain axonal tau localization. Intriguingly, the adult NMR tau protein is 88 kDa, much larger than 45–68 kDa tau expressed in other mammals. We propose that this 88 kDa tau protein may offer exceptional microtubule stability and neuroprotection against lifelong, elevated Aß.

1. Introduction

The microtubule-associated protein tau regulates several distinct neuronal processes including microtubule nucleation, polymerization, polarity, dynamics, spacing, and bundling. Tau's functional importance becomes evident in patients with microdeletions in the coding gene, microtubule-associated protein tau (*MAPT*). Although exceedingly rare, these mutations result in mental retardation and craniofacial abnormalities (Rovelet-Lecrux and Campion, 2012). Correspondingly, mice homozygous for *Mapt* deletion display long term-potentiation impairments resulting in cognitive deficits (Ahmed et al., 2014). Far more frequently, tau negative regulation occurs through posttranslational phosphorylation (Lindwall and Cole, 1984), and tau misprocessing, initiated primarily through hyperphosphorylation, coincides with neurodegeneration in a variety of disorders (e.g., Pick disease, argyrophilic grain disease, progressive supranuclear palsy, frontotemporal dementia, and chronic traumatic encephalopathy as well as Alzheimer's disease [AD]) (Spillantini and Goedert, 2013). Several studies have revealed exacerbated pathology in animal models upon genetically ablating *Mapt* including enhanced tauopathy (Ando et al., 2011), lysosomal dysfunction (Pacheco et al., 2009), parkinsonism (Lei et al., 2012) as well as Aß-associated axonal degeneration (Dawson et al., 2010) underscoring the importance of functional tau during situations of neurotoxic stress.

The vast majority of patients afflicted with tauopathies develop the diseases sporadically with age being the greatest risk factor. Incongruously, the most common animal models for these diseases are short-lived mice that do not naturally develop tauopathy, possibly because of their short life span. To overcome this shortcoming, induction of pathology is accomplished through genetic overexpression of mutant human gene variants. Fully penetrant AD-inducing mutations, accounting for <2% of all AD cases, occur in genes encoding proteins responsible for the accumulation of toxic, aggregation prone $Aß_{1-42}$. The concomitant misprocessing of tau, cognitive decline, behavioral changes, neuronal loss, and brain atrophy suggests that Aß, alone, may drive AD. Discovery of these familial AD mutations engendered the "Aß hypothesis" (Hardy and Higgins, 1992) that posits Aß accumulation is necessary and sufficient to drive AD pathogenesis and tauopathy. Many AD models expressing elevated Aß display cognitive impairments but do not develop appreciable tau pathology unless multiple transgenes (e.g.,

*Corresponding author at: Department of Physiology and The Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center at San Antonio, 15355 Lambda Drive, San Antonio, TX 78245, USA. Tel.: +1 210 562 5062; fax: 210 5625028.
E-mail address: buffenstein@uthscsa.edu (R. Buffenstein).

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mutant human tau, (Oddo et al., 2003)) are also expressed and neurodegeneration seldom occurs (Wirths and Bayer, 2010). Strikingly, despite successful pharmacologic amelioration of AD pathology and attenuation of cognitive decline over 300 times in such animal models, none of these promising preclinical findings have translated into effective and safe human therapies (Zahs and Ashe, 2010). The reasons, although not entirely clear, may reflect the modeling of the extremely rare heritable diseases in short-lived mammals, rather than addressing the more common sporadic nature of tauopathy and the use of model systems with life spans long enough to track pathogenesis.

Recently, we discovered naturally expressed, high levels of Aß in the brains of the naked mole-rat (NMR, *Heterocephalus glaber*), the longest-lived rodent known (Edrey et al., 2013). These mouse-sized rodents defy maximum life span predictions by living approximately 5 times longer than expected based on their body size and approximately 8 times longer than similar-sized mice. With a maximum life span of >30 years, they offer an exceptional perspective to investigate age-associated diseases. Many NMRs express higher brain Aß levels than do 3xTg-AD mice (Edrey et al., 2013), a well-characterized mouse model of AD pathology genetically manipulated to express high levels of this toxic peptide (Oddo et al., 2003). The NMR Aß peptide differs from that of human Aß by one amino acid, but both human and NMR Aß exert similar neurotoxicity to mouse cortical neurons in vitro (Edrey et al., 2013). NMRs, however, do not develop manifest plaques and seemingly maintain neuronal integrity. We wondered whether tau metabolism remained properly regulated despite >30 years of high levels of Aß peptide. Specifically, we investigated whether NMR tau protein was similar to human tau (e.g., sequence, size, expression, and phosphorylation), how it changed with age, and whether lifelong elevated levels of Aß altered tau misprocessing in NMRs. We assessed levels of total tau, phosphorylated tau, and known tau kinases throughout the NMR 30-year life span and compared adult brain histology with that of well-characterized 3xTg-AD mice.

2. Methods

2.1. Animals

Animal procedures were carried out in adherence to NIH, Federal, State, and Institutional guidelines at University of Texas Health Science Center San Antonio (University of Texas Health Science Center at San Antonio UTHSCSA; protocol #07123).

2.2. Tissue collection

Brains from 38 NMRs (<1 year) and 24 (>1 year) were collected. Animals were anesthetized by isoflurane inhalation then transcardially perfused with ice-cold phosphate-buffered saline (PBS), pH 7.4. Brains were immediately harvested, weighed, and sagitally bisected. One hemibrain was drop fixed in 10% zinc formalin for 48 hours, then transferred to PBS containing 0.02% sodium azide, and stored at 4 °C until further histologic processing. The other hemibrain was snap frozen in liquid nitrogen and stored at −80 °C for future biochemical analyses.

2.3. Tissue homogenization

For biochemistry, frozen hemibrains (minus cerebellum and brain stem) were slightly thawed on ice and mechanically homogenized with dounce and pestle in ice-cold buffer H (10 mM Tris HCl pH 7.4, 1 mM EGTA, 0.8 M NaCl, 10% sucrose) containing complete protease inhibitor (Roche, Basel, Switzerland) with or without phosphatase inhibitors (Invitrogen, Carlsbad, CA). Brain homogenates were centrifuged 14,000 rpm for 20 minutes at 4 °C. The supernatant was used for all further capillary electrophoresis. Protein concentration was determined using BCA assay (Pierce, Rockford, IL).

2.4. Capillary electrophoresis immunoassay

Capillary electrophoresis immunoblotting, or Simple Western analyses, were performed using the Simon and Wes platforms for tau and tau kinase levels, respectively, according to the manufacturer's protocol (ProteinSimple, Santa Clara, CA). This state of the art technology is considered more sensitive and quantitative than traditional Western blot immunoblotting, generating highly reproducible electropherograms with precise peaks and facilitating accurate molecular weight assessments (Creamer et al., 2014; Nguyen et al., 2011). In brief, brain homogenate was diluted to 6 μg (for Simon) or 1 μg (for Wes) in sample buffer and added to a master mix containing dithiothreitol and fluorescent molecular weight marker then heated at 95 °C for 5 minutes. The chemiluminescent substrate, HRP-conjugated secondary antibody, primary antibody, blocking reagent, samples, and separation and stacking matrices were dispensed into a 384-well plate. After plate loading, the separation electrophoresis and immunodetection steps took place in the capillary system and were fully automated. Simple Western analysis is carried out at room temperature and instrument default settings were used. Primary antibodies used the following: rabbit anti-total and phospho-GSK3ß Ser^{9}, total and phospho-ERK1/2 $Thr^{202/204}$ and $Thr1^{85/187}$, Cdk5, p35, and mouse anti-Tau46 (1:50) and mouse anti-GAPDH (1:100) (Cell Signaling, Danvers, MA). Dilutions of anti-tau antibodies are as follows: Tau1 (1:100); RD3, RD4, and Tau5 (1:50) (Millipore, Billerica, MA); mouse anti-CP13 and PHF1 (1:10) (generous gifts from Peter Davies); mouse anti-HT7 (1:50) (Pierce). Recombinant human tau was purchased (rPeptide, Bogart, GA). All antibodies were diluted with antibody diluent (ProteinSimple). The digital image was analyzed with Compass software (ProteinSimple v2.5), and the quantified data of the detected protein were reported as molecular weight. Protein densitometry was calculated by dividing the area under the curve of each protein of interest by area under the curve of GAPDH loading control.

2.5. Phosphatase assay

For removing posttranslational phosphorylation, brain homogenates were suspended in 0.8 μg/μL of NEBuffer 3 and incubated with 20U/μL of calf intestinal phosphatase (CIP) (New England Biolabs, Ipswich, MA) for 60 minutes at 37 °C. CIP-treated samples were compared with samples incubated in NEBuffer 3 without CIP.

2.6. Immunohistochemistry

Zinc formalin-fixed tissues were sectioned (30 μm thick) using a sliding vibratome and stored in 0.02% sodium azide in PBS until immunostaining was conducted. The endogenous peroxidase activity was quenched with 3% H_2O_2 in 10% methanol for 30 minutes. Tissue was incubated overnight at 4 °C with corresponding primary antibody. Sections were washed in tris-buffered saline and incubated in biotinylated secondary antibody for 1 hour at 20 °C. Sections were developed with diaminobenzidine substrate using the avidin-biotin horseradish peroxidase system (Vector Labs, Burlingame, CA, USA). Primary antibodies: HT7 (1:3000) and CP13 (1:2000). Images were obtained with a Zeiss camera. For each antibody, all tissues were immunostained simultaneously to eliminate batch-to-batch variability in antibody concentrations, incubation times, or chromogen exposure. Similarly, all images were acquired using identical settings to prevent light, exposure, or other subtle microscopy differences. As such, relative differences in immunostaining at the various ages can be compared with confidence.