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Amyloid beta and the longest-lived rodent: the naked mole-rat as a model for natural protection from Alzheimer's disease

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ABSTRACT

Amyloid beta (Aβ) is implicated in Alzheimer's disease (AD) as an integral component of both neural toxicity and plaque formation. Brains of the longest-lived rodents, naked mole-rats (NMRs) approximately 32 years of age, had levels of Aβ similar to those of the 3xTg-AD mouse model of AD. Interestingly, there was no evidence of extracellular plaques, nor was there an age-related increase in Aβ levels in the individuals examined (2–20+ years). The NMR Aβ peptide showed greater homology to the human sequence than to the mouse sequence, differing by only 1 amino acid from the former. This subtle difference led to interspecies differences in aggregation propensity but not neurotoxicity; NMR Aβ was less prone to aggregation than human Aβ. Nevertheless, both NMR and human Aβ were equally toxic to mouse hippocampal neurons, suggesting that Aβ neurotoxicity and aggregation properties were not coupled. Understanding how NMRs acquire and tolerate high levels of Aβ with no plaque formation could provide useful insights into AD, and may elucidate protective mechanisms that delay AD progression.

1. Introduction

Naked mole-rats (*Heterocephalus glaber*; NMRs) are mouse-size rodents that live more than 30 years in captivity and maintain good health for most of their long lives (Buffenstein, 2008). NMRs naturally exhibit 2 known risk factors for neurodegeneration, namely vitamin D deficiency (Buffenstein and Yahav, 1991) and high levels of oxidative stress (Andziak et al., 2006). Nevertheless, recent studies reveal unchanged gene expression (Kim et al., 2011) and sustained protein levels of key neurotrophic factors (Edrey et al., 2012) in the NMR brain during aging. Given the extreme longevity of NMRs, their naturally high levels of oxidative stress and low levels of vitamin D, we questioned whether NMRs accrue amyloid beta (Aβ), a cleaved small (38–43 amino acids) component of amyloid precursor protein (APP) that is causally implicated in Alzheimer's disease (AD). This common neurodegenerative disease is characterized by neuronal loss and senile plaques containing Aβ.

Several forms of Aβ exist, with the longer $A\beta_{1-42}$ peptide regarded as more aggregation-prone and harmful than the shorter forms (LaFerla et al., 2007). Indeed, increased production of $A\beta_{1-42}$ has been strongly implicated in AD (Citron et al., 1992). The precise role of Aβ in AD remains controversial; although Aβ is a key component of senile plaques, there is no correlation between plaques and impaired cognition (Arriagada et al., 1992; Giannakopoulos et al., 2003). Several studies suggest that extracellular insoluble aggregations of Aβ are not the culprits of AD symptoms. Rather, these, may be benign or even protective, removing from within the cell the more toxic soluble oligomeric Aβ aggregates (Klein et al., 2001; LaFerla et al., 2007). In vitro cell culture studies confirm this premise, for these oligomers are more neurotoxic than fibrillar Aβ (Pimplikar, 2009; Shankar et al., 2008).

Species differences in Aβ peptide sequences have been previously reported (Gotz and Ittner, 2008), and these vary in their in vitro toxicity and aggregation properties (Boyd-Kimball et al., 2004; Cecchi et al., 2007; Liu et al., 1999). Mice and rats have the same $A\beta_{1-42}$ sequence, which differs from human Aβ by 3 amino acids. This change in sequence causes a reduction in both the propensity of Aβ to self-aggregate and its degree of neurotoxicity (Boyd-Kimball et al., 2004), and may contribute to findings that mice/rats do not naturally develop AD-like symptoms. In this study, we hypothesized that extraordinarily long-lived NMRs would be useful models for human AD. Specifically, we asked whether NMRs showed an age-associated accrual of Aβ with concomitant plaque

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presence and asked whether the properties of the NMR Aβ peptide were similar to those of the human form.

2. Methods

2.1. Tissue collection and processing

Whole brains (minus cerebellum and brain stem) were harvested from NMRs (n = 39) of both sexes and various ages (2–29 years). These were flash-frozen in liquid nitrogen and stored in −80 °C until used in ELISAs and immunoblotting. Brains of 3xTg-AD mice served both as positive experimental controls and as a gauge with which to compare NMR $A\beta_{1-40}$ and $A\beta_{1-42}$ levels. Eight-month-old 3xTg-AD mice, albeit still young, show behavioral deficits as well as high levels of the soluble forms of Aβ (Billings et al., 2005; Oddo et al., 2003; Oddo et al., 2005; Oddo et al., 2008). Additional NMR brains were perfused and/or post-fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical analyses. In this case, 18 month-old 3xTg-AD (n = 4) mice were used in tandem as positive controls. Animal procurement and euthanasia 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. Sequencing of NMR Aβ

RNA was extracted from NMR brains (n = 5) by homogenization in TRI reagent (Sigma; St. Louis, MO), and RNA integrity was verified by agarose gel electrophoresis. cDNA was synthesized using the Masterscript kit (5 PRIME, Gaithersburg, MD). Following the manufacturer's instructions, 2 μg of RNA was used per reaction. Primers were designed based on the availability of sequences for other rodents as well as humans in GenBank, and encompassed the region 276 to 317 of the equivalent of human APP.

Forward: 5′-TCGACCAGGTTCTGGGTTGACAAA-3′;

Reverse: 5′-TTCGTAGCCGTTCTGCTGCATCTT-3′.

PCR reactions were subject to the following conditions: 1 minute denaturation cycle at 95 °C, 35 cycles each consisting of 1 minute at 95 °C, 30 seconds at 55.5 °C, and 1 minute at 72 °C, and a final extension step at 72 °C. The amplicons were cloned using One Shot TOPO10 chemically potent *Escherichia coli* cells (Invitrogen, Grand Island, NY). Plasmids were prepared by QIAprep Miniprep kits (Qiagen, Valencia, CA) and sequenced (Genewiz, South Plainfield, NJ). PCR fragments from each sample were sequenced for both strands, and these were compared to one another. In cases where mismatches occurred, the entire process was repeated until 100% homology was reached.

2.3. $A\beta_{1-42}$ aggregation

2.3.1. Kinetic assay

Human $A\beta_{1-42}$ (AnaSpec, Fremont, CA, catalog no. 20276), rodent Aβ (catalog no. 25381) and customized NMR Aβ (AnaSpec run no. 86396) consisting of 42 amino acids at the highest purity available (≥95%) were solubilized with 1.5% NH_4OH and diluted with phosphate-buffered saline (PBS) to a stock solution of 226 μmol/L. Aliquots were stored at −20 °C until use. The propensity to aggregate was based on a kinetic assay (Cecchi et al., 2007). $A\beta_{1-42}$ peptides representing all 3 species were incubated at a concentration of 226 μmol/L in PBS, pH 7.2 at 37 °C, and each sample was measured in triplicate in this kinetic assay. At regular time intervals, 10-μL aliquots of each sample were added to 490 μL of a solution containing 25 μmol/L thioflavin-T (ThT), 25 mmol/L phosphate buffer, pH 6.0. The steady-state fluorescence values of the resulting samples were measured at 25 °C using a spectrophotometer (Molecular Devices, Sunnyvale, CA). The excitation and emission wavelengths were 440 and 485 nm, respectively. All measured fluorescence values are given after subtracting the ThT fluorescence intensity measured in the absence of protein and normalized so that the final fluorescence intensity at the endpoint of the kinetic trace was 100% using the following equation: (initial/final) × 100/max.

2.3.2. Visualization by atomic force microscopy

Aliquots of synthetic NMR or human $A\beta_{1-42}$ (AnaSpec) were diluted to 1.13 μmol/L in ddH_2O. A 3-μL quantity of the samples was deposited on freshly cleaved mica, washed with ddH_2O, dried under a stream of nitrogen, and mounted in a Nanoscope IIIa microscope (Bruker/Veeco, Irvine, CA) equipped with an E scanning head-tapping mode. Imaging was performed in tapping mode in air, with TESP probes (Bruker/Veeco). Resonant frequency of the probes was 280 to 320 kHz, with 50 to 100 mV amplitude and a setpoint of 1.2 and 1.8V. Trace and retrace images were acquired for fields ranging from 0.49 μm^2 to 4 μm^2, with rates of 2.6 to 3 Hz. Standard flattening and plain-fit, and occasional removal of single aberrant scan lines were the only processing applied to raw images (Nanoscope III software, Bristol, UK). Grain analysis was performed with SPIP 5.1.11 (Image Metrology, Horsholm, Denmark), with automatically set baseline. The threshold of 0.4 nm was automatically set for the human peptide samples incubated for 48 hours. Grain analysis data collected from 3 0.49 μm^2 or 1 μm^2 fields in each case was further examined with OriginPro 8.6 (OriginLab Corp, Northampton, MA). The number of particles analyzed was as follows: human 770 (time 0); 529 (1 hour); 960 (48 hours); NMR 782 (0); 382 (1); 729 (48). The numerical values of areas covered by particles at the 0.4-nm threshold ("particle footprint" in nm^2) were delivered by the SPIP grain analysis. The areas are approximated by the closest polygon covering contour of the particle at a given height (threshold).

2.4. Neurotoxicity assessment on primary hippocampal neuron culture

Primary neurons were prepared from hippocampi of postnatal day 0 C57BL/6 mice. Cells were dispersed by incubation for 7 minutes at 37 °C in papain (Worthington Biochemical Corp., Lakewood, NJ) followed by trituration. Cell suspension was diluted in glial-conditioned neurobasal media supplemented with 1% B-27, 0.5 mmol/L glutamine, and 1x Insulin-Transferrin-Selenium-A supplement (Invitrogen). Neurons were plated on poly-D-lysine–coated 48-well plates at a density of 1.5×10^5 per well and used after culturing for 5 days. Neurons from each individual were equally dispersed among 3 wells that were treated with only media (control) or 10 μmol/L of either NMR or human $A\beta_{1-42}$ for 24 hours at 37 °C. Wells were treated with 1 mg/mL MTT in media for 2 hours, after, treatment with DMSO and read using a spectrophotometer (Molecular Devices) at 550 nm. Samples originating from the same individuals were compared to the controls from the same origin, and average fold decrease in cell viability was calculated.

2.5. Quantification of soluble and insoluble Aβ

Hemibrains not containing cerebellum or brain stem were dissected from 39 non-littermate NMRs (2–29 years old) and 3xTg-AD mice (n = 3). Samples were sonicated and homogenized and Aβ levels were measured by a sensitive sandwich enzyme-linked immunosorbent assay (ELISA) (Oddo et al., 2005). Brains were homogenized in protein extraction reagent (T-PER buffer,