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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 (A β) 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 A β 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 A β levels in the individuals examined (2–20+ years). The NMR A β 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 A β was less prone to aggregation than human A β . Nevertheless, both NMR and human A β were equally toxic to mouse hippocampal neurons, suggesting that A β neurotoxicity and aggregation properties were not coupled. Understanding how NMRs acquire and tolerate high levels of A β with no plaque formation could provide useful insights into AD, and may elucidate protective mechanisms that delay AD progression.

Keywords

Alzheimer's disease; Amyloid beta; Naked mole-rat; Aggregation; Neuronal toxicity; 3xTg-AD mice; *Heterocephalus glaber*

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)

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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 ($A\beta$), 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 $A\beta$.

Several forms of $A\beta$ 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 $A\beta$ in AD remains controversial; although $A\beta$ 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 $A\beta$ 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 $A\beta$ 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 $A\beta$ (Pimplikar, 2009; Shankar et al., 2008).

Species differences in $A\beta$ 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 $A\beta$ by 3 amino acids. This change in sequence causes a reduction in both the propensity of $A\beta$ 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 $A\beta$ with concomitant plaque presence and asked whether the properties of the NMR $A\beta$ 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 $A\beta$ (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 A β

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 μ g 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 β_{1-42} aggregation

2.3.1. Kinetic assay—Human A β_{1-42} (AnaSpec, Fremont, CA, catalog no. 20276), rodent A β (catalog no. 25381) and customized NMR A β (AnaSpec run no. 86396) consisting of 42 amino acids at the highest purity available ($\sim 95\%$) were solubilized with 1.5% NH₄OH and diluted with phosphate-buffered saline (PBS) to a stock solution of 226 μ mol/L. Aliquots were stored at -20 °C until use. The propensity to aggregate was based on a kinetic assay (Cecchi et al., 2007). A β_{1-42} peptides representing all 3 species were incubated at a concentration of 226 μ mol/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- μ L aliquots of each sample were added to 490 μ L of a solution containing 25 μ mol/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) $\times$ 100/max.

2.3.2. Visualization by atomic force microscopy—Aliquots of synthetic NMR or human A β_{1-42} (AnaSpec) were diluted to 1.13 μ mol/L in ddH₂O. A 3- μ L quantity of the samples was deposited on freshly cleaved mica, washed with ddH₂O, 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 1× 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 $\mu\text{mol/L}$ of either NMR or human $\text{A}\beta_{1-42}$ 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 $\text{A}\beta$

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 $\text{A}\beta$ 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, Pierce, Rockford, IL) containing protease (Roche Complete Mini) and phosphatase inhibitors (Calbiochem, Billerica, MA, 1:100). The homogenized mixes were sonicated to shear the DNA and centrifuged at 4 °C for 1 hour at $100,000 \times g$. The supernatant was collected and stored in -80 °C until use as the soluble fraction. The pellet was rehomogenized in 70% formic acid and centrifuged as above. The supernatant was similarly stored, and is referred to as the insoluble fraction. Proteins from the soluble fraction were loaded directly onto microplates, and formic acid fractions were diluted 1:20 in neutralization buffer (1 mol/L Tris base/0.5 mol/L NaH_2PO_4 dibasic) before loading. Maxisorp immunoplates (Nalge Nunc) were coated with monoclonal antibody 20.1, a specific antibody against $\text{A}\beta_{1-16}$ (a kind gift from F. LaFerla, University of California–Irvine) in coating buffer (0.1 mol/L NaCO_3 , pH 9.6), and blocked with 3% bovine serum

albumin. Synthetic A β standards (Bachem, Torrance, CA) were defibrillated by dissolving in hexafluoroisopropanol (HFIP) at 1 mg/mL, and the HFIP evaporated with a stream of N₂. The defibrillated A β was dissolved in DMSO at 1 mg/mL. Standards of both A β 1–40 and A β 1–42 were made in antigen capture buffer (20 mmol/L NaH₂PO₄/2 mmol/L ethylenediaminetetraacetic acid (EDTA)/0.4 mol/L NaCl/0.5 g of CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)/1% bovine serum albumin, pH 7.0) and loaded onto microplates in duplicate. Samples were loaded in duplicate and incubated overnight at 4 °C. Plates were washed and probed with either horseradish peroxidase–conjugated anti–A β 35–40 (MM32–13.1.1 for A β 40) or anti–A β 35–42 (MM40–21.3.4 for A β 42) overnight at 4 °C. 3,3',5,5'-tetramethylbenzidine was used as the chromagen, and the reaction was stopped with the addition of 30% O-phosphoric acid and read at 450 nm on a plate reader (Molecular Devices). Values were normalized by protein content.

2.6. C-terminal fragments

Four brain homogenates were used for immunoblots. Gels were transferred to nitrocellulose membranes and these were treated with antibodies for CT-20 (1:2500, Calbiochem #17160) and actin (1:5000; mouse, Calbiochem) for normalization purposes. Both C-terminal fragments were observed and quantified from the same membranes, and therefore the ratio between the 2 was compared. Data were analyzed using ImageJ (National Institutes of Health) and appear as mean $\pm$ SEM.

2.7. A β plaques

Immunohistochemical assessments of A β distribution and analysis included paraffin-embedded sagittal sections (5- μ m thick; n = 28) from brains of 2- to 29-year-old breeding (n = 16) and nonbreeding (n = 10) NMR that were either perfused with 4% paraformaldehyde or were not perfused. In addition, free-floating coronal sections of the oldest individuals (20+ years) were used (50 μ m thick; n = 3). Sections were treated with the antibodies 4G8 and 6E10 (Covance, Princeton, NJ, 1:1000) with or without formic acid as an antigen retrieval step (reviewed in LaFerla et al., 2007). Some free-floating sections were also treated with a 1% w/v solution of Thioflavin-S alongside 18-month-old 3xTg-AD mice to visualize mature plaques.

Negative controls were treated identically except that the primary antibody was not included. Free-floating sections of 18-month 3xTg-AD mice, known to acquire AD pathology served as positive controls. Visualization was performed with the Axio A1 Microscope (Zeiss) and images taken at various magnifications using AxioVision 4.7.2 software (Zeiss). Thioflavin-S samples were visualized with Leica Microsystems.

3. Results

3.1. Unique sequence of NMR A β

NMR A β differs from humans by only 1 amino acid: at position 13, human A β contains histidine, whereas in NMRs it is arginine (H13R; Fig. 1A). NMR A β differs from mouse/rat A β by 2 amino acids (R5G, Y10F), whereas human A β sequence differs from the mouse/rat

sequence by 3 amino acids (R5G, Y10F, H13R). Nucleotide and protein sequences were submitted to GenBank (GenBank IDs: HM446016.1 and ADL59579.1, respectively).

3.2. Low aggregation propensity for NMR A β ₁₋₄₂

Aggregation propensity was assessed using synthetic peptides with the same amino acid sequences as NMR A β ₁₋₄₂, mouse A β ₁₋₄₂, and human A β ₁₋₄₂ in a kinetic thioflavin-T (ThT) fluorescence assay. NMR A β , like mouse A β (Fig. 1B), showed a similar propensity to aggregate (10.76 ± 0.33 A.U., and 11.34 ± 0.84 A.U., respectively), which was significantly lower than that of human A β (14.33 ± 0.47 A.U.; analysis of variance (ANOVA), $F = 10.4$, $p = 0.011$).

Distinct aggregation properties for both human and NMR A β ₁₋₄₂ were confirmed using atomic force microscopy (AFM) at different time points. At time 0, A β particles from both sources showed similar particle footprints; most particle areas were up to 15 nm^2 with ~60% of the particles in that small size category (Fig. 2A, Fig. 3A and B). Human A β ₁₋₄₂ showed a clear tendency to form much bigger aggregates. After 1 hour of incubation, the fraction of human A β ₁₋₄₂ small particles declined (~43%), whereas NMR A β ₁₋₄₂ showed considerably more small particles (~55%; Fig. 2B, Fig. 3C and D), and these remained at that size even at 48 hours (~52%; Fig. 2C, Fig. 3E and F). After 48 hours, the fraction of small particles in human A β ₁₋₄₂ had further declined (26%) and several distinct footprint size classes corresponding to further aggregation stages were evident. At this time point, the largest particles found in human A β ₁₋₄₂ reached 3000 nm^2 (Fig. 3E), whereas the largest particles in NMR samples never attained 500 nm^2 (Fig. 3F). The total fraction of polymerized peptide was similar at time 0 ($0.76\% \pm 0.003\%$) for the human and ($0.81\% \pm 0.002\%$) for NMR particles (t test, $p = 0.9$; Fig. 2D). After 48 hours, pronounced species' differences were evident with $12.75\% \pm 0.007\%$ of the area covered by polymerized human A β ₁₋₄₂, and only $0.95\% \pm 0.001\%$ by the NMR peptides (t test, $p = 0.00001$). Interestingly, the NMR polymerized peptide showed no time-dependent increase in aggregation relative to earlier time points.

3.3. NMR A β ₁₋₄₂ has similar neurotoxicity to human A β ₁₋₄₂

A β ₁₋₄₂ exposure is reportedly more toxic to neurons than A β ₁₋₄₀. Therefore, we compared the neurotoxicity properties of both human and NMR A β ₁₋₄₂ by measuring the fraction of mouse primary hippocampal neurons surviving exposure to a supraphysiological dose ($10 \text{ }\mu\text{mol/L}$) of either NMR or human A β ₁₋₄₂. Both forms of A β ₁₋₄₂ induced very similar levels of cell death, with 18% of all neurons in cell culture dying 24 hours post-treatment (Fig. 3G). Clearly both NMR and human A β ₁₋₄₂ have similar toxic properties of A β ₁₋₄₂ irrespective of the 1-amino acid difference in sequence.

3.4. High endogenous levels of A β in NMRs

Levels of the soluble A β ₁₋₄₀ were highly variable in young NMRs ($864.30 \pm 253.79 \text{ pg A}\beta/\text{mg protein}$, $n = 15$). Given this high variability, although the average levels were 4-fold greater in the NMR than in the 3xTg-AD mouse model ($194.56 \pm 8.87 \text{ pg A}\beta/\text{mg protein}$, $n = 3$), data were not statistically different (t test, $p = 0.26$; Fig. 4A). Average soluble levels of A β ₁₋₄₂ (Fig. 4B) were similar (t test, $p = 0.92$) between NMRs ($248.22 \pm 70.53 \text{ pg A}\beta/\text{mg}$

protein, $n = 15$) and 3xTg-AD mice (232.43 ± 32.13 pg A β /mg protein, $n = 3$). Average levels of insoluble A β_{1-40} (Fig. 4C), although considerably lower than the soluble form, were also similar (t test, $p = 0.69$) between NMRs (36.89 ± 15.89 pg A β /mg protein, $n = 14$) and 3xTg-AD mice (51.81 ± 30.98 pg A β /mg protein, $n = 3$). One NMR sample was omitted from this mean, as it (508.96 pg A β /mg protein) was more than 2 standard deviations from the average. Average insoluble A β_{1-42} levels (Fig. 4D) in NMRs (60.25 ± 21.82 pg A β /mg protein; $n = 15$) and 3xTg-AD mice (75.21 ± 19.52 pg A β /mg protein; $n = 3$) were similar (t test, $p = 0.77$). For NMRs, levels of soluble A β_{1-40} and A β_{1-42} were both significantly higher than their respective insoluble counterpart (t test, $p = 0.004$ and $p = 0.017$, respectively).

3.5. Levels of A β do not increase with age

Regardless of the A β moiety (soluble and insoluble A β_{1-40} and A β_{1-42}), NMR levels ($n = 39$) were within a range similar to those of young (8-month-old, $n = 3$) 3xTg-AD mice (Fig. 5A–D). Moreover, there were no significant correlations between age and levels of A β . Neither soluble A β_{1-40} (Fig. 5A) nor A β_{1-42} (Fig. 5B) increased with age ($R^2 = 0.0162$, $p = 0.441$; $R^2 = 0.0539$, $p = 0.155$, respectively). Similarly, levels of insoluble A β_{1-40} (Fig. 5C) were unchanged with age ($R^2 = 0.0003$, $p = 0.914$). Levels of the insoluble A β_{1-42} (Fig. 5D) doubled with every doubling of aging ($y = 23.536x + 1.314$; $R^2 = 0.136$, $p = 0.022$), with the correlation (r value) accounting for 36.8% of the observed variability. However, this relationship was anchored by 1 very high value in the oldest cohort, and if this outlier is not included in the linear regression analyses, this relationship is no longer observed ($y = 12.70x + 108.96$; $R^2 = 0.094$, $p = 0.112$).

3.6. A β production does not change with age

APP processing did not significantly change with age (Fig. 6A and B). C83 and C99 are the C-terminal fragments that remain after APP processing. The former is a footprint of the non-amyloidogenic pathway, whereas the latter implies the occurrence of an amyloidogenic event. Neither C83 (Fig. 6A) nor C99 (Fig. 6B) changed significantly among young (2-year-old), older (10–15 years), and oldest (20+ years) NMRs (ANOVA, $F = 2.572$, $p = 0.171$ and $F = 2.149$, $p = 0.212$, respectively). In all age groups, the ratio between C83 and C99, however, suggests that there are more non-amyloidogenic events than amyloidogenic ones.

3.7. No evidence of A β plaques

Tissues were stained with 2 different antibodies that are traditionally used to detect human (6E10) and mouse (4G8) A β . In both cases, based on the primary sequence of NMR A β , there appeared to be no recognition artifacts, and neither antibody revealed the existence of extracellular plaques in NMRs (Fig. 6D and F). The study included paraffin-embedded thin sections (5 μ m) and free-floating thick sections (50 μ m). The latter were also stained with thioflavin-S, which does not rely on antibody recognition and would fluoresce upon binding to mature plaques. This method also did not reveal any indications of plaques in the aged NMR brain (not shown). 3xTg-AD mice were used as positive controls for 6E10 staining (Fig. 6E and G) and thioflavin-S (not shown), and clearly detected A β plaques using both

techniques. These data reveal that despite high levels of A β , histological assessments of the brains of animals as old as 29 years do not reveal A β plaques.

4. Discussion

In this study we assessed whether extraordinarily long-lived NMRs showed signs of A β accrual and damage as is commonly the case in humans with AD. Both humans and NMRs live 4-to 5-fold longer than expected by body size and are considered long-lived species. We and others have shown that the NMRs' extreme longevity goes hand in hand with sustained good health, and that NMRs show both a delayed and attenuated aging profile with compression of morbidity restricted to only the last few years of life (Edrey et al., 2011A; Edrey et al., 2012; Lambert et al., 2007; Pérez et al., 2009; Ungvari et al., 2008). We questioned whether these long-lived rodents live long enough to accrue high levels of A β and senile plaques, and whether NMR A β properties differ substantially from those of humans. We therefore characterized the sequence, aggregation propensity, and neurotoxicity properties of NMR A β and assessed whether NMRs have detectable levels of A β , and evaluated whether these accrue with age.

The sequence of NMR A β (GenBank ID: ADL59579.1) is, for the most part, identical to both mouse and human A β , and differs from that of mouse/rat A β at only 2 amino acid sites and from human A β by only 1 amino acid (Fig. 1A). This small species difference between humans and NMRs nevertheless appeared to be sufficient to alter in vitro A β aggregation but not toxicity properties. The single amino acid substitution in the NMR (compared to human A β) reduced the tendency of A β to aggregate, confirming earlier chemical studies that changes to certain amino acids had profound effects on aggregation properties (Boyd-Kimball et al., 2004; Cecchi et al., 2007; Hilbich et al., 1991; Liu et al., 1999). That a single amino acid sequence change can have an impact on the functional properties of A β is fascinating, and must reflect 3-dimensional structural differences. Human A β has 2 adjacent histidines (at positions 13 and 14), and these are proposed as important metal binding domains that would promote aggregation, neurotoxicity (Atwood et al., 1998; Liu et al., 1999; Tickler et al., 2005), or, conversely, would serve as sites for A β to bind and internalize itself into neurons (Diaz et al., 2006; Poduslo et al., 2010). The change in 1 amino acid at position 13, a key site for Cu(II) binding (Atwood et al., 1998) is missing in the NMR A β_{1-42} compared to human A β_{1-42} . This suggests that in the NMR, Cu(II) binding is likely deficient (either in amount or affinity). Given the high levels of oxidative stress and similar neurotoxicity properties in NMRs, Cu(II) may not play as prominent a role in A β metabolism. Interestingly, the *Octodon degu* shares the same A β sequence as the NMR, and it does, in fact, show plaques and AD-like symptoms Inestrosa et al., 2005, Van Groen et al., 2011, so it is difficult to conclude the role of this change from the data that we have gathered here.

Cecchi et al. (2007) reported that the propensity for A β aggregation differs also by depending upon the total peptide size. For example, A β_{1-40} and A β_{1-42} show different aggregation properties; A β_{1-42} assemblies reach high aggregation states within minutes, contrary to A β_{1-40} . Based on the size of the aggregates as viewed with AFM, NMR A β_{1-42} formed both significantly smaller and fewer aggregates than the more abundant, larger fibril

human aggregates (Figs. 2,3). Once human A β monomeric peptides (~4 kDa) are produced, they readily form dimers, oligomers, and fibrils. The oligomers, not the large fibrils, are currently regarded as the key culprits for damaging and killing cells in the central nervous system (Fodero-Tavoletti et al., 2011).

NMR and human A β_{1-42} in this study induced similar degrees of neurotoxicity (Fig. 3G). Given that human A β_{1-42} takes less than 72 hours to form fibrils, it is possible that species' differences in toxicity would be evident if human A β_{1-42} were allowed to aggregate before toxicity experiments. In this case, we would predict that because NMR A β does not aggregate into large fibrils, NMR A β may be more toxic than the human form. If this were the case, our findings would support the premise that insoluble fibrils/plaques may be protective, removing the toxin from the cell before it can essentially do much harm.

Unlike both human and mouse A β , the NMR A β_{1-42} showed divergent aggregation and neurotoxicity properties, revealing that these are not necessarily codependent but, rather, are uncoupled. Interestingly, the NMR A β sequence is identical to that of a small South American rodent, the *Octodon degus* (degu) (Inestrosa et al., 2005) that belongs to the same taxonomic suborder of rodents, the hystricognathae. A β deposits have been reported in the brains of old degus. Moreover, by 6 years of age, degus had symptoms and behavior reminiscent of AD-like neurodegeneration (Ardiles et al., 2012, Inestrosa et al., 2005, van Groen et al., 2008). Clearly, therefore, the NMR and degu A β sequence has the potential to be both neurotoxic and aggregation prone in vivo.

Surprisingly, on average, young adult NMR brains had high amounts of both soluble and insoluble A β_{1-40} and A β_{1-42} . These were within range of those measured in 8-month-old 3xTg-AD mice, which are specifically designed to express high levels of human APP to model AD-like pathology. The large variance evident in the NMR data set may be reflective of the fact that these are second-generation captive, non-manipulated individuals, originating from different wild colonies. It is unlikely that this is reflective of housing or dietary conditions, as all individuals in our colony are maintained under identical conditions and fed the same diet. It may also reflect the high variance in a natural population toward developing AD-like pathology, similar to what occurs in humans. In contrast, the low variation observed within the 3xTg-AD data set reflects the fact that these mouse mutants are deliberately bred to maintain a specific genetic identity and are thereby considered robust models for the study of familial AD. To date, there is no information regarding age-related brain pathology (e.g., strokes or compromises to the blood-brain barrier) in NMRs. Housing conditions and diets were identical in all individuals, and although all of the animals chosen for this study were healthy, it is possible that our data set may include individual variation due to life history. For example, our data set included both breeding and non-breeding individuals, which could introduce variation resulting from differences in sex and stress hormones between these groups (Clarke and Faulkes, 1997; Faulkes and Abbott, 1993).

Because oxidative stress is implicated as a key risk factor for A β accumulation, the high levels of A β observed in relatively young NMRs may be linked to earlier findings of high levels of oxidative stress in our captive colony (Andziak et al., 2006) and divergent individual cytoprotective responses to this artificial "hyperoxic" milieu (Edrey et al.,

2011B). NMRs have led a strictly subterranean existence since the early Miocene and have evolved to tolerate the hypoxic (~8%–15% oxygen) conditions encountered by living in large groups in poorly ventilated underground burrows. Life in captivity thus presents a pronounced oxidative stress to these animals living in a gaseous atmosphere of 20.9% oxygen normally found above ground. Clearly, NMRs are extremely resilient and capable of tolerating these stressors. Living permanently in dark sealed burrows, this species is chronically vitamin D deficient, with undetectable levels of circulating 24-hydroxyvitamin D3 and very low levels of calcitriol (Buffenstein and Yahav, 1991). Vitamin D is thought to play a role in the removal of A β from the brain (Masoumi et al., 2009; Smolders et al., 2011), and deficiency has been implicated as a risk factor for AD. Both oxidative damage and vitamin D status may therefore contribute to the observed high variability and high levels of A β in the brains of captive young NMRs.

Despite the observed high levels of A β in brain tissues, there were no signs of plaques even in a 29-year-old animal (Fig. 6D and F). This finding was most surprising, as the levels of A β were comparable to those observed in an AD mouse model. Furthermore, degus with the same A β sequence show signs of plaques and cognitive deficits even in middle age (Ardiles et al., 2012; Inestrosa et al., 2005; van Groen et al., 2011). The NMR is therefore a rare species in that, unlike other long-lived mammals (e.g., canines, primates), it does not show age-related pathology reminiscent of AD. In this regard, NMRs seem to be a natural model for studying the mechanisms that protect against an AD-like phenotype with age.

Given that NMRs endogenously produce high levels of A β at a young age, yet live more than 2 decades with these high levels, it appears that this species is exceedingly tolerant of high levels of A β _{1–42} and has evolved mechanisms to counter its neurotoxic effects. We have previously reported that NMRs have high levels of the neurotrophic factor Neuregulin 1 (NRG-1) known to maintain neuronal integrity, which remain elevated throughout life (Edrey et al., 2012). NRG-1 may serve NMRs from age-related and other neuronal impairments (e.g., A β -induced damage) and contribute to the observed attenuated aging profile in the NMR brain. As such, this species may prove to be an exciting new animal model with which to investigate the mechanisms that may protect against AD-like pathology.

It is widely accepted that AD pathology takes considerable time to develop, and that short-lived species may die of other causes before AD-like pathology may present. Given that NMRs live more than 3 decades, it is more likely that these rodents have sufficient time to develop some or all of pathology associated with AD. The absence of age-associated signs of plaques concurs with our earlier findings of “negligible senescence” in NMR behavior, physiology, and biochemistry of the visceral tissues (Buffenstein, 2008), as well as unchanged gene expression in the NMR brain with age (Kim et al., 2011). Indeed, there were no significant age-related changes in amyloidogenic A β production in the brain and the non-amyloidogenic pathway (C83, non-amyloidogenic remnant) continues to predominate over the amyloidogenic pathway (C99, amyloidogenic remnant), even in old age. Transcriptome analysis of 4 –year-old and 20-year-old brains concurs with this finding, for no age-related changes in any of the enzymes involved in the cleavage of APP to form the C-fragments (Kim et al., 2011) were evident.

A β is correlated with the severity of AD; however, levels of A β , at least in NMRs, may not be causally linked to AD progression. This may be due to protective mechanisms that prevent the manifestation of harmful effects of A β (e.g., antioxidants) (Baruch-Suchodolsky and Fischer, 2009) or A β may be a symptom of the disease and not a key causative player. Understanding the mechanisms that protect the NMR brain from the harmful effects of A β may reveal novel insights into putative translational strategies to protect against the age-associated progression into AD-like pathology.

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References

- Andziak B, O'Connor TP, Qi W, DeWaal EM, Pierce A, Chaudhuri AR, Van Remmen H, Buffenstein R. High oxidative damage levels in the longest-living rodent, the naked mole-rat. *Aging Cell*. 2006; 5:463–471. [PubMed: 17054663]
- Ardiles AO, Tapia-Rojas CC, Mandal M, Alexandre F, Kirkwood A, Inestrosa NC, Palacios AG. Postsynaptic dysfunction is associated with spatial and object recognition memory loss in a natural model of Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* 2012; 109:13835–13840. <http://dx.doi.org/10.1073/pnas.1201209109>. [PubMed: 22869717]
- Arriagada P, Growdon J, Hedley-Whyte E, Hyman B. Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology*. 1992; 42:631–639. [PubMed: 1549228]
- Atwood CS, Moir RD, Huang X, Scarpa RC, Bacarra NM, Romano DM, Hartshorn MA, Tanzi RE, Bush AI. Dramatic aggregation of Alzheimer abeta by Cu(II) is induced by conditions representing physiological acidosis. *J. Biol. Chem.* 1998; 273:12817–12826. [PubMed: 9582309]
- Baruch-Suchodolsky R, Fischer B. Abeta40, either soluble or aggregated, is a remarkably potent antioxidant in cell-free oxidative systems. *Biochemistry*. 2009; 48:4354–4370. [PubMed: 19320465]
- Billings LM, Oddo S, Green KN, McLaugh JL, LaFerla FM. Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron*. 2005; 45:675–688. [PubMed: 15748844]
- Buffenstein R. Negligible senescence in the longest living rodent, the naked mole-rat: insights from a successfully aging species. *J. Comp. Physiol. B*. 2008; 178:439–445. [PubMed: 18180931]
- Buffenstein R, Yahav S. Cholecalciferol has no effect on calcium and inorganic phosphorus balance in a naturally cholecalciferol-deplete subterranean mammal, the naked mole rat (*Heterocephalus glaber*). *J. Endocrinol.* 1991; 129:21–26. [PubMed: 1851511]
- Cecchi C, Fiorillo C, Baglioni S, Pensalfini A, Bagnoli S, Nacmias B, Sorbi S, Nosi D, Relini A, Liguri G. Increased susceptibility to amyloid toxicity in familial Alzheimer's fibroblasts. *Neurobiol. Aging*. 2007; 28:863–876. [PubMed: 16781020]
- Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I, Selkoe DJ. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature*. 1992; 360:672–674. [PubMed: 1465129]
- Boyd-Kimball D, Sultana R, Mohammad-Abdul H, Butterfield DA. Rodent Abeta(1–42) exhibits oxidative stress properties similar to those of human Abeta(1–42): implications for proposed mechanisms of toxicity. *J. Alzheimers Dis.* 2004; 6:515–525. [PubMed: 15505374]
- Clarke F, Faulkes C. Dominance and queen succession in captive colonies of the eusocial naked mole-rat, *Heterocephalus glaber*. *Proc. Biol. Sci.* 1997; 264:993–1000. [PubMed: 9263466]
- Diaz JC, Linnehan J, Pollard H, Arispe N. Histidines 13 and 14 in the Abeta sequence are targets for inhibition of Alzheimer's disease Abeta ion channel and cytotoxicity. *Biol. Res.* 2006; 39:447–460. [PubMed: 17106577]

- Edrey YH, Casper D, Huchon D, Gelfond J, Mele J, Kristan DM, Nevo E, Buffenstein R. Sustained high levels of neuregulin-1 in the longest-lived rodents: a key determinant of rodent longevity. *Aging Cell*. 2012; 11:213–222. [PubMed: 22103690]
- Edrey YH, Hanes M, Pinto M, Mele J, Buffenstein R. Successful aging and sustained good health in the naked mole rat: a long-lived mammalian model for biogerontology and biomedical research. *ILAR J*. 2011A; 52:41–53. [PubMed: 21411857]
- Edrey YH, Park TJ, Kang H, Biney A, Buffenstein R. Endocrine function and neurobiology of the longest-living rodent, the naked mole-rat. *Exp. Gerontol*. 2011B; 46:116–123. [PubMed: 20888895]
- Faulkes CG, Abbott DH. Evidence that primer pheromones do not cause social suppression of reproduction in male and female naked mole-rats (*Heterocephalus glaber*). *J. Reprod. Fertil*. 1993; 99:225–230. [PubMed: 8283442]
- Fodero-Tavoletti MT, Villemagne VL, Rowe CC, Masters CL, Barnham KJ, Cappai R. Amyloid-beta: the seeds of darkness. *Int. J. Biochem. Cell. Biol*. 2011; 43:1247–1251. [PubMed: 21601003]
- Giannakopoulos P, Herrmann F, Bussière T, Bouras C, Kövari E, Perl D, Morrison J, Gold G, Hof P. Tangle and neuron numbers, but not amyloid load, predict cognitive status in Alzheimer's disease. *Neurology*. 2003; 60:1495–1500. [PubMed: 12743238]
- Gotz J, Ittner LM. Animal models of Alzheimer's disease and frontotemporal dementia. *Nat. Rev. Neurosci*. 2008; 9:532–544. [PubMed: 18568014]
- Hilbich C, Kisters-Woike B, Reed J, Masters CL, Beyreuther K. Human and rodent sequence analogs of Alzheimer's amyloid beta A4 share similar properties and can be solubilized in buffers of pH 7.4. *Eur. J. Biochem*. 1991; 201:61–69. [PubMed: 1915378]
- Inestrosa NC, Reyes AE, Chacón MA, Cerpa W, Villalón A, Montiel J, Merabachvili G, Aldunate R, Bozinovic F, Aboitiz F. Human-like rodent amyloid- β -peptide determines Alzheimer pathology in aged wild-type *Octodon degu*. *Neurobiol Aging*. 2005; 26:1023–1028. [PubMed: 15748782]
- LaFerla FM, Green KN, Oddo S. Intracellular amyloid-beta in Alzheimer's disease. *Nat. Rev. Neurosci*. 2007; 8:499–509. [PubMed: 17551515]
- Lambert AJ, Boysen HM, Buckingham JA, Yang T, Podlasky A, Austad SN, Kunz TH, Buffenstein R, Brand MD. Low rates of hydrogen peroxide production by isolated heart mitochondria associate with long maximum life-span in vertebrate homeotherms. *Aging Cell*. 2007; 6:607–618. [PubMed: 17596208]
- Liu ST, Howlett G, Barrow CJ. Histidine-13 is a crucial residue in the zinc ion-induced aggregation of the A β peptide of Alzheimer's disease. *Biochemistry*. 1999; 38:9373–9378. [PubMed: 10413512]
- Masoumi A, Goldenson B, Ghirmai S, Avagyan H, Zaghi J, Abel K, Zheng X, Espinosa-Jeffrey A, Mahanian M, Liu PT, Hewison M, Mizwickie M, Cashman J, Fiala M. 1 α ,25-dihydroxyvitamin D3 interacts with curcuminoids to stimulate amyloid-beta clearance by macrophages of Alzheimer's disease patients. *J. Alzheimers Dis*. 2009; 17:703–717. [PubMed: 19433889]
- Kim EB, Fang X, Fushan AA, Huang Z, Lobanov AV, Han L, Marino SM, Sun X, Turanov AA, Yang P, Yim SH, Zhao X, Kasaikina MV, Stoletzki N, Peng C, Polak P, Xiong Z, Kiezun A, Zhu Y, Chen Y, Kryukov GV, Zhang Q, Peshkin L, Yang L, Bronson RT, Buffenstein R, Wang B, Han C, Li Q, Chen L, Zhao W, Sunyaev SR, Park TJ, Zhang G, Wang J, Gladyshev VN. Genome sequencing reveals insights into physiology and longevity of the naked mole rat. *Nature*. 2011; 479:223–227. [PubMed: 21993625]
- Klein WL, Krafft GA, Finch CE. Targeting small A β oligomers: the solution to an Alzheimer's disease conundrum? *Trends Neurosci*. 2001; 24:219–224. [PubMed: 11250006]
- Oddo S, Caccamo A, Green KN, Liang K, Tran L, Chen Y, Leslie FM, LaFerla FM. Chronic nicotine administration exacerbates tau pathology in a transgenic model of Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* 2005; 102:3046–3051. [PubMed: 15705720]
- Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kaye R, Metherate R, Mattson MP, Akbari Y, LaFerla FM. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular A β and synaptic dysfunction. *Neuron*. 2003; 39:409–421. [PubMed: 12895417]

- Oddo S, Caccamo A, Tseng B, Cheng D, Vasilevko V, Cribbs DH, LaFerla FM. Blocking Abeta42 accumulation delays the onset and progression of tau pathology via the C terminus of heat shock protein70-interacting protein: a mechanistic link between Abeta and tau pathology. *J. Neurosci.* 2008; 28:12163–12175. [PubMed: 19020010]
- Pérez VI, Buffenstein R, Masamsetti V, Leonard S, Salmon AB, Mele J, Andziak B, Yang T, Edrey Y, Friguet B, Ward W, Richardson A, Chaudhuri A. Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat. *Proc. Natl. Acad. Sci. U.S.A.* 2009; 106:3059–3064. [PubMed: 19223593]
- Pimplikar SW. Reassessing the amyloid cascade hypothesis of Alzheimer's disease. *Int. J. Biochem. Cell. Biol.* 2009; 41:1261–1268. [PubMed: 19124085]
- Poduslo JF, Gilles EJ, Ramakrishnan M, Howell KG, Wengenack TM, Curran GL, Kandimalla KK. HH domain of Alzheimer's disease Abeta provides structural basis for neuronal binding in PC12 and mouse cortical/hippocampal neurons. *PLoS One.* 2010; 5:e8813. [PubMed: 20098681]
- Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA, Regan CM, Walsh DM, Sabatini BL, Selkoe DJ. Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat. Med.* 2008; 14:837–842. [PubMed: 18568035]
- Smolders J, Moen SM, Damoiseaux J, Huitinga I, Holmoy T. Vitamin D in the healthy and inflamed central nervous system: access and function. *J. Neurol. Sci.* 2011; 311:37–43. [PubMed: 21862439]
- Tickler AK, Smith DG, Ciccotosto GD, Tew DJ, Curtain CC, Carrington D, Masters CL, Bush AI, Cherny RA, Cappai R, Wade JD, Barnham KJ. Methylation of the imidazole side chains of the Alzheimer disease amyloid-beta peptide results in abolition of superoxide dismutase-like structures and inhibition of neurotoxicity. *J. Biol. Chem.* 2005; 280:13355–13363. [PubMed: 15668252]
- Ungvari Z, Buffenstein R, Austad SN, Podlutzky A, Kaley G, Csiszar A. Oxidative stress in vascular senescence: lessons from successfully aging species. *Front. Biosci.* 2008; 13:5056–5070. [PubMed: 18508570]
- van Groen T, Kadish I, Popovic N, Popovic M, Caballero-Bleda M, Bano-Otalora B, Vivanco P, Rol MA, Madrid JA. Age-related brain pathology in *Octodon degu*: blood vessel, white matter and Alzheimer-like pathology. *Neurobiol. Aging.* 2011; 32:1651–1661. [PubMed: 19910078]

Fig. 1.

Slight species differences in A β sequence lead to marked differences in A β aggregation properties. Note positions 5, 10, and 13 of the A β sequence, where species differences between human and mouse A β are evident. Only position 13 differs between human and NMR (histidine and arginine, respectively) (A). Nevertheless, NMR and mouse/rat A β have a lower propensity to aggregate than the human form (ANOVA, $p = 0.011$), as measured by the change in kinetic fluorescence of ThT (B). Data are presented as mean $\pm$ SEM for triplicates of each sample.

Fig. 2.

Grain analysis data reveal that aggregation properties depend on sequence and incubation conditions. Grain analysis data extracted from 3 fields for both human and NMR Aβ₁₋₄₂ examined with AFM OriginPro 8.6 (OriginLab Corp., Northampton, MA) reveal significant differences between both samples as represented by distribution of footprint parameter (area covered by a particle). At time 0, no significant changes are evident between human and NMR Aβ₁₋₄₂. (t test, $p = 0.9$) (A). After 1 hour incubation at room temperature, human Aβ₁₋₄₂ aggregation is beginning to diverge from the NMR sample (t test, $p = 0.69$) (B). After 48 hours' incubation at 37 °C, human and NMR Aβ₁₋₄₂ show 2 discrete patterns of aggregation (C). Percentage of total area coverage by aggregates (particles) differs drastically (t test, $p = 0.00001$) between human (hatched bars) and NMR (white bars) after 48 hours of incubation. The coverage after 0 or 1 hour does not differ significantly (D). Data presented as mean $\pm$ SEM.

Fig. 3.

Aggregation and neurotoxicity properties of NMR A β are uncoupled. AFM images reveal differences in polymerization of human and NMR A β_{1-42} . The presented raw topography images of 750 $\times$ 750-nm fields were zoomed in from 1- μm^2 fields. Human A β_{1-42} (A) and NMR A β_{1-42} (B) at time 0 show a similar pattern of aggregation. After 1 hour at 37 °C, a divergent pattern begins to emerge (C and D). After 48 hours' incubation at 37 °C, a striking difference is evident between both samples with greater aggregation evident in the human A β_{1-42} (E) compared to the NMR A β_{1-42} (F). The gray scale represents the height of

particles, with black representing the background (lowest areas) and light shades representing A β polymers. Neurotoxicity of NMR A β is similar to that of human A β (G). Mouse primary neurons were pretreated with or without (control) 10 μ mol/L of either form of A β , and cell viability was assessed with MTT assay 24 hours after treatment. A decline of 18% cell viability was apparent when A β was used, regardless of the sequence used (NMR or human). Data presented are mean $\pm$ SEM, as normalized to controls.

Fig. 4.

Levels of Aβ are detectable in young NMRs and are comparable to those of young 3xTg-AD mice. Young NMRs (2–9 years old; $n = 15$; gray-shaded circles) show levels of Aβ similar to those of young (8-month-old; $n = 3$; open squares) 3xTg-AD mice. Six of the samples for soluble Aβ₁₋₄₀ had undetectable levels (A). Soluble Aβ₁₋₄₀ and soluble Aβ₁₋₄₂ (B) were not significantly different from those of 8-month-old 3xTg-AD mice (t test, $p = 0.26$ and $p = 0.92$, respectively). Levels of insoluble Aβ₁₋₄₀ were also similar, with 1 NMR outlier as calculated by having levels more than 2 SDs higher than the average is shown on the graph. It was not included to calculate average levels or statistical analysis (t test, $p = 0.69$) (C). Levels of insoluble Aβ₁₋₄₂ were also similar to those of 3xTg-AD mice (t test, $p = 0.77$) (D). All data points obtained in our experiments are represented, and the average per species appears as a bar.

Fig. 5.

Levels of Aβ do not increase with age in NMRs. Levels of NMR (gray circles) soluble Aβ₁₋₄₀ (A) and Aβ₁₋₄₂ (B) did not increase with age (2–26 years; gray circles $y = -16.48x + 1274.25$; $R^2 = 0.0162$, $p = 0.44$; $y = 14.819x + 264.187$; $R^2 = 0.0539$, $p = 0.155$, respectively). Similarly, levels of insoluble Aβ₁₋₄₀ (C) did not increase with age ($y = -0.806x + 166.723$; $R^2 = 0.0003$, $p = 0.914$). In contrast, although insoluble Aβ₁₋₄₂ (D) levels in young animals are comparatively very low, because of 1 very high value in the aged group, a significant positive correlation with age, was apparent ($y = 23.53x + 1.314$; $R^2 = 0.136$, $p = 0.022$, respectively); however, if this 1 outlier is ignored, there was no significant relationship ($y = 12.70x + 108.96$; $R^2 = 0.094$, $p = 0.112$). In all cases, NMR levels are within similar range to those of young (8-month-old) 3xTg-AD mice (open squares). All data points obtained in our experiments are represented; $n = 39$ for NMRs, $n = 3$ for 3xTg-AD mice.

Fig. 6.

No age-related change in APP processing with no evidence for senile plaques. C83 and C99 are the C-terminal fragments that remain after APP processing. The former is a footprint of the non-amyloidogenic pathway, whereas the latter implies an amyloidogenic event. Neither C83 (A) nor C99 (B) changed significantly among young (2 years), older (10–15 years), and the oldest (20+ years) NMRs (ANOVA $F = 2.572$, $p = 0.171$; $F = 2.149$, $p = 0.212$, respectively). A representative immunoblot of C83 and C99 is shown (C, top) with the corresponding loading control (C, bottom). In all age groups, the ratio between C83 and

C99, however, suggests that there are more non-amyloidogenic events than amyloidogenic ones, providing some protection against A β production. Immunostaining with 6E10 antibody does not reveal plaques in NMRs. Note that some intraneuronal staining appears; staining with 4G8 antibody revealed the same pattern and is therefore not shown here. No plaques detected in low (D) or high (F) magnification of a 29-year-old NMR. For control purposes, immunohistochemistry was performed in tandem with an 18-month-old 3xTg-AD mouse. Low (E) and high (G) magnification clearly shows A β plaques (arrows).