Age-related decreases in SYN levels associated with increases in MAP-2, apoE, and GFAP levels in the rhesus macaque prefrontal cortex and hippocampus

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Abstract Loss of synaptic integrity in the hippocampus and prefrontal cortex (PFC) may play an integral role in age-related cognitive decline. Previously, we showed age-related increases in the dendritic marker microtubule associated protein 2 (MAP-2) and the synaptic marker synaptophysin (SYN) in mice. Similarly, apolipoprotein E (apoE), involved in lipid transport and metabolism, and glial fibrillary acidic protein (GFAP), a glia specific marker, increase with age in rodents. In this study, we assessed whether

these four proteins show similar age-related changes in a nonhuman primate, the rhesus macaque. Free-floating sections from the PFC and hippocampus from adult, middle-aged, and aged rhesus macaques were immunohistochemically labeled for MAP-2, SYN, apoE, and GFAP. Protein levels were measured as area occupied by fluorescence using confocal microscopy as well as by Western blot. In the PFC and hippocampus of adult and middle-aged animals, the levels of SYN, apoE, and GFAP immunoreactivity were comparable but there was a trend towards higher MAP-2 levels in middle-aged than adult animals. There was significantly less SYN and more MAP-2, apoE, and GFAP immunoreactivity in the PFC and hippocampus of aged animals compared to adult or middle-aged animals. Thus, the age-related changes in MAP-2, apoE, and GFAP levels were similar to those previously observed in rodents. On the other hand, the age-related changes in SYN levels were not, but were similar to those previously observed in the aging human brain. Taken together, these data emphasize the value of the rhesus macaque as a pragmatic translational model for human brain aging.

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Introduction

Decline in cognitive function with age has been reported in humans, nonhuman primates, and rodents

(Zyzak *et al.* 1995; Albert and Moss 1999; Moss *et al.* 1999; Benice *et al.* 2006), yet the biological cause of age-related cognitive decline is not fully understood. The areas of interest for age-related cognitive decline in humans and nonhuman primates are the prefrontal cortex (PFC) and hippocampus, brain regions highly associated with learning and memory (Paulesu *et al.* 1993; Levy and Goldman-Rakic 1999). During normal aging, the hippocampus is one of the first structures to decline (Geinisman *et al.* 1995; Winocur and Gagnon 1998), observed behaviorally as a change in performance in hippocampal dependent cognitive tasks in rodents, nonhuman primates, and humans (Rapp *et al.* 1997; Maguire and Cipolotti 1998; Rosenbaum *et al.* 2001). It has been commonly hypothesized that cognitive decline results from a loss of neurons within the brain. However, there is convincing evidence that the number of neurons does not significantly change during normal aging (Rapp and Gallagher 1996; Merrill *et al.* 2000; Keuker *et al.* 2003). Consequently, a more plausible alternative hypothesis is that a change of the integrity of the synapses mediating learning and memory contributes to the decline in cognitive function.

Microtubule associated protein 2 (MAP-2) and synaptophysin (SYN) are synaptic proteins associated with learning and memory. MAP-2 is a dendritic protein responsible for the assembly of the microtubules in the dendrites and aids in dendritic plasticity (Harada *et al.* 2002). SYN is a presynaptic vesicle protein located in axons associated with synaptic vesicles and is responsible for formation of synapses (Tarsa and Goda 2002). SYN levels also positively correlate with cognitive function in rodents (Dawson *et al.* 1999; Mulder *et al.* 2004). Reports from rodent studies indicate that the levels of MAP-2 and SYN immunoreactivity are dependent on age, area of the brain, and amount of cognitive testing (Chauhan and Siegel 1997; Calhoun *et al.* 1998; Di Stefano *et al.* 2001; Shapiro and Whitaker-Azmitia 2004; Himeda *et al.* 2005; Benice *et al.* 2006; Adams *et al.* 2008; Minkeviciene *et al.* 2008; Prieto-Gomez *et al.* 2008).

Apolipoprotein E (apoE), a protein with an integral role in lipid transport and metabolism, can also alter synaptic activity. Age-related increases of apoE in the rat hippocampus positively correlates with cognitive impairment (Kadish *et al.* 2009). In addition, the human apoE ϵ 4 allele is linked to an increased risk of

developing Alzheimer's disease (AD), a disease known to decrease the function of the hippocampus in humans (Gosche *et al.* 2002). ApoE appears to play a mechanistic role as plaques associated with AD are constructed of β -amyloid (A β) and apoE proteins (Nakamura *et al.* 1996; Burns *et al.* 2003). Similarly, interactions of increased apoE levels with alpha synuclein in the brain can lead to neuronal degeneration (Gallardo *et al.* 2008).

Glial fibrillary acidic protein (GFAP), an astrocyte specific marker, increases with age in mice (Kohama *et al.* 1995), rats, and humans (Nichols *et al.* 1993). An increase of GFAP has also been reported in the subcortical white matter of elderly rhesus macaques, compared to younger animals (Sloane *et al.* 2000). Furthermore, in aged cynomolgus macaques, GFAP immunoreactive processes are present around A β and apoE plaques (Nakamura *et al.* 1996). Interestingly, high expression levels of GFAP have also been associated with AD (Nichols *et al.* 1993; Hol *et al.* 2003; Si *et al.* 2004), with higher GFAP expression found in subjects with apoE ϵ 4 allele (Overmyer *et al.* 1999).

Although an age-related change in immunoreactivity of MAP-2, SYN, apoE, and GFAP has been reported in rodents and humans, these proteins have not been extensively studied in the nonhuman primate. One report indicated that no difference in MAP-2 and SYN was observed between young adult (5-year) and old (18-year) nonhuman primates; however, there were only two old animals in the study and the analysis was performed in the pallidum-nigral region (Fukuda *et al.* 2005). However, since rhesus macaques in captivity can live longer than 30 years (Uno *et al.* 1996), the current study includes much older animals and examined cortical regions linked to cognitive function. Additionally, studying older animals could provide more translational insight into the elderly human brain and bridge the translational efforts of rodent and human research (Chauhan and Siegel 1997; Calhoun *et al.* 1998; Benice *et al.* 2006; Kadish *et al.* 2009).

In the present study, immunohistochemistry and Western blot were used to assess age-related changes in MAP-2, SYN, apoE, and GFAP in the PFC and hippocampus from a heterogeneous population of adult, middle-aged, and elderly rhesus macaques. In addition, some of the brain sections were double-labeled immunohistochemically for apoE and GFAP

or apoE and A β , to assess potential colocalization of these proteins.

Methods

Animals

Post-mortem brains were obtained from adult (5–7 years; $n=5$; 2 male, 3 female), middle-aged (10–15 years; $n=5$; 2 male, 3 female), and aged (23–31 years; $n=5$; 2 male, 3 female) rhesus macaques (*Macaca mulatta*), through the Tissue Distribution Program at ONPRC/OHSU. At death, the average weight was 6.0 ± 0.7 kg for adult, 6.5 ± 0.8 kg for middle-aged, and 8.5 ± 0.7 kg for aged animals. In general, all animals were in overall good health during their lifetime. One female in the adult group and one female in the middle-aged group had been ovariectomized. None of the animals used in this study were behaviorally tested. All of the brains were perfused with saline at room temperature followed by ice-cold 4% paraformaldehyde. After perfusion, the brains were cut into blocks, briefly post-fixed in paraformaldehyde solution, cryoprotected, then frozen and stored in -80°C .

Histology

Frozen brains were sectioned at 25 μm (PFC coronal sections) or 40 μm (hippocampal coronal sections) using a Microm HM 400 microtome (GMI, Ramsey, MN, USA). For PFC assessment of MAP-2, SYN, apoE, and GFAP, six sections (2.0 mm between each section) were selected from each brain for each protein. Likewise, for hippocampus assessment of MAP-2, SYN, apoE, and GFAP, 10 sections (1.2 mm between sections) were selected from each brain for each protein. Free-floating sections were washed with fresh PBS followed by a 10% Normal Goat Serum (NGS; MAP-2/SYN) or Normal Sheep Serum (NSS; apoE/GFAP/apoE + GFAP/apoE + β -amyloid) in PBS + 0.3% Triton for 1 h. Next, sections were incubated in 0.3% Triton + 1% NGS/NSS in PBS with the primary antibody (anti-MAP-2 raised in mouse, Sigma-Aldrich, 0.5 $\mu\text{g}/\text{ml}$; anti-SYN raised in mouse, Sigma-Aldrich, 0.5 $\mu\text{g}/\text{ml}$; anti-apoE raised in goat, Calbiochem, La Jolla, CA, USA, 1:4,000; anti-GFAP raised in rabbit, Sigma-Aldrich, 1.0 $\mu\text{g}/\text{ml}$;

anti- β -amyloid [A β 1–42] raised in mouse, Sigma-Aldrich, 1.0 $\mu\text{g}/\text{ml}$) overnight at room temperature. Two sets of tissue sections were double-labeled for apoE and GFAP or apoE and β -amyloid. For double-labeled sections, primary antibodies were added at the same time for a single primary antibody incubation. Sections were washed three times in fresh PBS, for 10 min each wash. They were then incubated in 0.3% Triton + 1% NGS/NSS in PBS with a secondary antibody (MAP-2: anti-mouse FITC raised in donkey, Jackson, 15 $\mu\text{g}/\text{ml}$; SYN: anti-mouse Texas Red raised in donkey, Jackson, 15 $\mu\text{g}/\text{ml}$; apoE: anti-goat FITC raised in donkey, Jackson, 15 $\mu\text{g}/\text{ml}$; GFAP: anti-rabbit Texas Red raised in donkey, Jackson, 15 $\mu\text{g}/\text{ml}$; β -amyloid: anti-mouse Texas Red raised in donkey, Jackson, 15 $\mu\text{g}/\text{ml}$) for 2 h at room temperature. Control sections were processed as described above, but without the primary antibody. Sections were mounted on slides (Fisher plus, Fisher Scientific, Pittsburg, PA, USA), dehydrated, and coverslipped with DPX Mountant (Sigma-Aldrich) and imaged with a Confocal Microscope (Olympus Spinning Disk Confocal, Center Valley, PA, USA).

Imaging and quantification

Images of the brain sections were acquired with Slidebook (Olympus) from layers 2–4 in the dorsal, lateral, medial, orbitofrontal, dorsal area 46, and ventral area 46 of the PFC, as well as the CA1, CA3, dentate gyrus (DG) of the hippocampus and layers 2–4 of the entorhinal cortex (EhC). Immunofluorescence was analyzed from six images per section for each section in the PFC set and four images per section for each of the sections in the hippocampus set. Images were taken of the same areas of the PFC or hippocampus from each brain section, using identical confocal parameters, including exposure time. To further address intensity issues, background intensity was determined using representative images for each brain section. Furthermore, all images were taken in a z-plane of 3 μm . A maximum intensity collapsed image was used for quantification. Area occupied by fluorescence immunoreactivity was calculated for each image. Subsequently, for each tissue set, the area occupied by fluorescence immunoreactivity in all images was averaged for the PFC and hippocampus. Quantification of the number of cells expressing GFAP was also performed.

Western blot analysis

A second set of post-mortem brain tissue, different from the brains used for immunohistochemistry but similar in age, were obtained from the PFC and hippocampus of adult (5–9 years; $n=4$; 2 males, 2 females), middle-aged (11–18 years; $n=4$; 2 males, 2 females), and aged (23–31 years; $n=4$; 2 males, 2 females) male and female rhesus macaques, through the Tissue Distribution Program at ONPRC/OHSU. At death, the average weight was 7.9 ± 1.2 kg for adult, 7.2 ± 0.5 kg for middle-aged, and 7.6 ± 0.7 kg for aged animals. All animals were in overall good health during their lifetime. One female in the adult group was ovariectomized. None of the animals used in this study were behaviorally tested. All of the brains were flushed with 1 L of saline solution kept at room temperature. After perfusion, the brains were dissected, frozen, and stored at -80°C .

The tissues were homogenized using 1 ml of RIPA buffer (Pierce Pharmaceuticals, Rockford, IL, USA) and Halt protease inhibitor (Pierce Pharmaceuticals). Homogenized tissues were spun at $12,000\times g$ for 15 min, and the supernatants were transferred to new tubes and kept for analysis. Protein concentrations were determined using a Nanodrop Spectrometer (NanoDrop ND-1000).

For each sample and lane, 40 μg of protein was used for Western blot analysis. Proteins were first denatured for 15 min by boiling them in a solution of Laemmli's buffer containing 2-mercaptoethanol (Sigma-Aldrich) and samples were loaded onto pre-prepared gels (Criterion Bio-Rad Ready Gels, 4–15% Tris-HCl, 18-well). In addition to the tissue samples, one lane was used for the Precision Plus Protein Western Standard (Bio-Rad). Gels were run using a Bio-Rad Power Pac for 60 min at 175 V. Subsequently, proteins were transferred to PVDF membranes for 90 min at 30 V.

Once proteins were transferred to the membranes, the membranes were placed in blocking buffer [5% dry-milk in PBS containing 0.5% Tween-20 (TBS-Tween)] for 1 h. Membranes were washed with the TBS-Tween buffer (4×5 min) and incubated with the dry-milk buffer containing β -actin antibody (Santa Cruz Biotechnology, raised in mouse, 0.5 $\mu\text{g}/\text{ml}$) for 12 h at 4°C . Membranes were washed with TBS-Tween buffer (4×5 min) and incubated in the dry-

milk buffer containing the secondary antibody for detecting the primary antibody against β -actin, donkey anti-mouse-HRP (Santa Cruz, 1 $\mu\text{g}/\text{ml}$) for 1 h and the secondary antibody for detecting the protein ladder, Precision protein StrepTactin-HRP (Bio-Rad; 5 μl). Subsequently, the membranes were incubated in Immun-Star HRP (Bio-Rad) for 5 min and imaged (FluorChem Q, Alpha Innotech, San Leandro, CA). After imaging, the antibodies were stripped from the membranes using Restore stripping buffer (Pierce Pharmaceuticals) for 5 min at room temperature. Membranes were re-blocked in blocking buffer (5% dry-milk TBS-Tween) for 1 h. Membranes were then washed in TBS-Tween buffer (4×5 min) and incubated with one primary antibody (anti-MAP-2 raised in mouse, Sigma-Aldrich, 1 $\mu\text{g}/\text{ml}$; anti-SYN raised in mouse, Sigma-Aldrich, 1 $\mu\text{g}/\text{ml}$; anti-apoE raised in goat, Calbiochem, 1:4,000; anti-GFAP raised in rabbit, Sigma-Aldrich, 1.0 $\mu\text{g}/\text{ml}$) for 12 h at 4°C . Membranes were washed in TBS-Tween buffer (4×5 min) and incubated in the corresponding secondary antibody (anti-mouse-HRP, 1 $\mu\text{g}/\text{ml}$ for MAP-2 and SYN; anti-goat-HRP raised in donkey, 1 $\mu\text{g}/\text{ml}$ for apoE; anti-rabbit-HRP raised in donkey, 1 $\mu\text{g}/\text{ml}$ for GFAP; Santa Cruz Biotechnology) for 1 h. Blots were incubated with Immun-star HRP for 5 min and imaged. Blots were probed a maximum of three times, only being stripped twice. β -actin was used as one of the probes for each membrane as the loading control. Pixel densities of the β -actin, MAP-2, SYN, apoE, and GFAP bands were determined using FluorChem Q software. Background levels were automatically determined by the software using upper- and lower-edge interpolation. Semiquantification using densitometry for the β -actin MAP-2, SYN, apoE, and GFAP bands were measured for each sample. The results are presented as a ratio between the antigen of interest (MAP-2/SYN/apoE/GFAP) and β -actin bands.

Statistical analysis

Statistical analyses were performed with SPSS 16.0 (SPSS, Chicago, IL USA). Area occupied by fluorescence of MAP-2, SYN, apoE, and GFAP immunoreactivity, and GFAP cell number was analyzed by one-way ANOVAs with Bonferroni's post-hoc tests. For the semiquantification of immunoblots, results for each

brain area were analyzed using one-way ANOVAs and Bonferroni's post-hoc tests. Significance was considered at $P < 0.05$.

Results

Microtubule associated protein 2

In the PFC, there was an interaction between the levels of MAP-2 immunoreactivity and age [$F(2, 312) = 9.8$, $P < 0.001$; Figs. 1 and 2a]. Although MAP-2 immunoreactivity was not significantly different between the adult and middle-aged rhesus macaques ($P = 1.0$), it was significantly greater in the aged macaques compared to the adult and middle-aged macaques ($P < 0.001$). There was no difference in MAP-2 immunoreactivity between the specific regions of the PFC ($P = 0.9$). As described below in detail, western blot analyses revealed similar patterns for all markers as the fluorescence immunohistochemistry (i.e. See also western results, Fig. 3).

Similarly, in the hippocampus, there was an effect of age on MAP-2 immunoreactivity [$F(2, 272) = 38.6$, $P < 0.01$; Figs. 4 and 8a]. There was a progressive age-

dependent increase in MAP-2 immunoreactivity in hippocampus with all age groups showing significant differences in areas occupied by fluorescence ($P < 0.01$). There was no difference in MAP-2 immunoreactivity the CA1, CA3, and DG of the hippocampus or the EhC within the age groups ($P = 0.2$).

Synaptophysin

There was an effect of age on SYN immunoreactivity in the PFC [$F(2, 312) = 5.9$, $P < 0.01$; Figs. 1 and 2b]. While there was no difference in SYN immunoreactivity in the adult and middle-aged rhesus macaque ($P = 1.0$), SYN immunoreactivity was significantly lower in the aged than the adult and middle-aged macaques. No difference in SYN immunoreactivity was observed between the specific regions of the PFC ($P = 0.6$).

In the hippocampus, there was an effect of age on SYN immunoreactivity [$F(2, 272) = 4.24$, $P < 0.01$; Figs. 5 and 8b]. Although no difference was observed in SYN immunoreactivity between the adult and middle-aged rhesus macaques ($P = 1.0$), SYN immunoreactivity was significantly lower in the aged macaques ($P = 0.01$). No difference was observed in SYN immunoreactivity between the CA1, CA3, and DG of the hippocampus or the EhC ($P = 0.08$).

Apolipoprotein E

In the PFC, there was an effect of age on the amount of apoE immunoreactivity [$F(2, 312) = 7.3$, $P < 0.01$; Figs. 1 and 2c]. ApoE immunoreactivity was not significantly different between the adult and middle-aged monkeys ($P > 0.6$), but was significantly greater in the aged macaques ($P < 0.001$). Interestingly, apoE immunoreactivity in the aged rhesus macaques was concentrated in clusters, whereas the immunoreactivity in the adult and middle-aged rhesus macaques was located in cells and around vessels (Fig. 1). ApoE immunoreactivity was not different between the subregions of the PFC ($P = 0.6$).

Likewise, in the hippocampus, there was an age-related increase in immunoreactivity [$F(2, 272) = 62.7$, $P < 0.01$; Figs. 6 and 8c]. While there was no difference in apoE immunoreactivity between the adult and middle-aged macaques ($P = 1.0$), apoE immunoreactivity was significantly greater in the aged macaques ($P < 0.001$). Similar to the PFC, apoE

Fig. 1 Representative photomicrographs of MAP-2, SYN, apoE, and GFAP immunoreactivity from PFC of adult, middle-aged, and aged rhesus macaques. White arrow indicate GFAP cell clusters in the aged animal. Scale bar is 30 μm

Fig. 2 Age-related change in immunoreactivity in the PFC. **a** MAP-2 immunoreactivity, **b** SYN immunoreactivity, **c** apoE immunoreactivity, and **d** GFAP immunoreactivity. *** $P < 0.001$, ** $P < 0.01$ compared to both adult and middle-aged animals

immunoreactivity was concentrated in clusters in the aged macaques, whereas, in the younger animals, apoE was limited to a few cells and around blood vessels (Fig. 6). Area occupied by fluorescence was not significantly different between the subregions of the hippocampus ($P = 0.5$).

GFAP

The number of cells expressing GFAP was not significantly different between adult, middle-aged, and aged rhesus macaques in either the PFC or hippocampus ($P > 0.16$). However, there was a significant increase in immunoreactivity of GFAP in both the PFC and hippocampus ($P < 0.01$).

In the PFC, there was an effect of age on the amount of GFAP immunoreactivity [$F(2, 312) = 19.27$, $P < 0.001$; Figs. 1 and 2d]. GFAP immunoreactivity was not different between the adult and middle-aged monkeys ($P > 0.4$), but it was significantly greater in the aged macaques ($P < 0.001$). GFAP immunoreactivity in the aged rhesus macaques was concentrated in groups of cells, whereas the immunoreactivity in the adult and middle-aged rhesus macaques was located in individual cells (Fig. 1). GFAP immunoreactivity was not different between the subregions of the PFC ($P = 0.2$).

Likewise, in the hippocampus, there was an overall interaction between age and GFAP immunoreactivity [$F(2, 272) = 6.15$, $P < 0.01$; Figs. 7 and 8d]. Although

Fig. 3 Age-related changes in protein expression in the PFC measured by Western blot analysis. Representative images of Western blot and β-actin control for all antigens; lane 1 is from

adult tissue, lane 2 is from middle-aged tissue, and lane 3 is from aged tissue in all images. **a** MAP-2, **b** SYN, **c** apoE, and **d** GFAP. * $P<0.05$, ** $P<0.01$ compared to adult and middle-aged animals

there was no difference in GFAP immunoreactivity between the adult and middle-aged macaques ($P=1.0$), GFAP immunoreactivity was significantly greater in the aged macaques ($P<0.01$). Similar to the PFC, GFAP immunoreactivity was concentrated in

groups of cells in the aged macaques, whereas, in the younger animals, it was limited to individual cells. GFAP immunoreactivity was not significantly different between the subregions of the hippocampus ($P=0.5$).

Fig. 4 Representative photomicrographs of MAP-2 immunoreactivity from the hippocampus CA1, CA3, dentate gyrus (DG), and entorhinal cortex (EhC) of adult, middle-aged, and aged rhesus macaques. Scale bar is 30 μ m

GFAP and apoE colocalization

At all ages examined, there was little colocalization of GFAP and apoE in both the PFC and hippocampus. In the adult (Fig. 9a), middle-aged (not shown), and

Fig. 5 Representative photomicrographs of SYN immunoreactivity from respective areas of the hippocampus CA1, CA3, DG, and EhC of adult, middle-aged, and aged rhesus macaques. Scale bar is 30 μ m

Fig. 6 Representative photomicrographs of apoE immunoreactivity from respective areas of the hippocampus CA1, CA3, DG, and EhC of adult, middle-aged, and aged rhesus macaques. Scale bar is 30 μ m

aged animals (Fig. 9b, c), there was minimal overlap (yellow) of GFAP (red) and apoE (green), indicating little colocalization of the two proteins. In the aged animals, GFAP immunoreactivity was also frequently

Fig. 7 Representative photomicrographs of GFAP immunoreactivity from respective areas of the hippocampus CA1, CA3, DG, and EhC of adult, middle-aged, and aged rhesus macaques. White arrows identify examples of clusters of GFAP positive cells in the aged animals. Scale bar is 30 μ m

Fig. 8 Bar graphs representing MAP-2, SYN, apoE, and GFAP immunoreactivity in the CA1, CA3, DG, and EhC. **a** MAP-2 immunoreactivity. **b** SYN immunoreactivity. **c** apoE immuno-

reactivity. **d** GFAP immunoreactivity. *** $P < 0.001$, ** $P < 0.01$, compared to adult and middle-aged animals and # $P < 0.01$ compared to adult animals

found concentrated in groups of cells surrounding apoE clusters (Fig. 9c).

A β and apoE colocalization

No A β immunoreactivity was observed in the adult and middle-aged rhesus macaques, yet it was observed in clusters in the aged rhesus macaques. All apoE clusters were colocalized with A β immunoreactivity (Fig. 9e). However, not all A β -positive clusters were also apoE positive (Fig. 9d).

Western blot analysis

In the PFC, MAP-2 [$F(2, 11) = 16.8$; $P = 0.01$; Fig. 3a] and apoE [$F(2, 11) = 5.2$; $P = 0.03$; Fig. 3c] expression was significantly greater in the aged rhesus macaques compared to the adult and middle-

aged animals. A trend of increased GFAP ($P = 0.09$; Fig. 3d) was also observed. Conversely, SYN expression was significantly less in the aged rhesus macaques compared to the adult and middle-aged animals [$F(2, 11) = 4.5$; $P = 0.04$; Fig. 3b]. No significant difference was observed between the adult or middle-aged animals for any of the four antigens.

A similar pattern was observed in the hippocampus, with expression of MAP-2 [$F(2, 11) = 6.9$; $P = 0.01$; Fig. 10a], apoE [$F(2, 11) = 9.9$; $P = 0.005$; Fig. 10c], and GFAP [$F(2, 11) = 4.9$; $P = 0.04$; Fig. 10d] being significantly greater in the aged rhesus macaques compared to the adult and middle-aged animals. SYN expression was significantly less in the aged rhesus macaques compared to the adult and middle-aged animals [$F(2, 11) = 8.0$; $P = 0.01$; Fig. 10b]. No significant difference was observed

Fig. 9 Representative photomicrographs of colocalization of apoE and GFAP and apoE and A β . **a** Photomicrograph from adult rhesus macaque demonstrating little overlap of GFAP and apoE from the PFC. **b** Photomicrograph from an aged rhesus macaque from the PFC. GFAP immunoreactivity (red) and apoE immunoreactivity (green) in the same image. Yellow areas are the overlap of GFAP and apoE immunoreactivity. **c** White boxed area from **b** enlarged to demonstrate the lack of colocalization of GFAP and apoE. White arrow indicates apoE

immunoreactivity in close proximity, but not overlapping with GFAP immunoreactivity. Furthermore, GFAP grouping around apoE positive clusters are observed. **d** Photomicrograph from an aged rhesus macaque of an A β cluster that is not apoE positive from the PFC. **e** Photomicrograph from an aged rhesus macaque of an A β and apoE positive cluster from the PFC. Overlapping of A β and apoE immunoreactivity shows yellow. Scale bar is 30 μ m

between the adult or middle-aged animals for any of the four antigens.

Discussion

A loss of synaptic activity is hypothesized to contribute to age-related decline of cognitive function (Masliah *et al.* 1993; Liu *et al.* 1996). Rodent studies have been equivocal, with reports of an age-related increase of SYN (Himeda *et al.* 2005; Benice *et al.* 2006), no change in SYN with increasing age (Calhoun *et al.* 1998), and an age-related decrease of SYN (Adams *et al.* 2008) in the hippocampus. The differences between these studies could be a result of cognitive testing or experimental manipulation of subjects. On the other hand, human studies indicate that MAP-2, apoE, and GFAP increase in the hippocampus with age while SYN decreases with age (Nichols *et al.* 1993; Hesse *et al.* 1999; Mukaetova-Ladinska *et al.* 2000; Eastwood *et al.* 2006). Similar to human studies, we show an age-related increase of MAP-2, GFAP, and apoE, as well as a decrease of SYN in the rhesus macaque hippocampus. The observed increase in MAP-2 levels might compensate for the age-related decrease in SYN levels. Additionally,

the increase in GFAP levels could be a compensatory mechanism for the increase in apoE levels.

MAP-2 has been previously reported to be a sensitive marker for age-related changes in rodents (Benice *et al.* 2006; Peister *et al.* 2006). Here, we demonstrate a significant increase of MAP-2 immunoreactivity with increased age between the adult, middle-aged, and aged animals in the hippocampus of rhesus macaques, but not in the PFC. Conversely, no difference was observed between the adult and middle-aged animals in the SYN, apoE, or GFAP immunoreactivity in either the PFC or hippocampus. Therefore, MAP-2 is a marker for the early age-related changes in the hippocampus than SYN, apoE, or GFAP. Furthermore, the limbic system might be more sensitive to the effect of age than the neocortex.

In the aged animals, immunoreactivity of apoE was concentrated in clusters in both the PFC and the hippocampus. In rodents, elevated apoE levels correlate with impaired cognitive function (Kadish *et al.* 2009). Increased apoE expression resulting from high dietary cholesterol in humans is also associated with an increased incidence of AD (Petanceska *et al.* 2003). Nonhuman primates show an age-related decline in performance on tasks associated with PFC and hippocampal

Fig. 10 Age-related changes in protein expression in the hippocampus measured by Western blot analysis. **a** MAP-2, **b** SYN, **c** apoE, and **d** GFAP. ** $P < 0.01$, * $P < 0.05$ compared to adult and middle-aged animals

function (Rapp and Amaral 1991; Rapp and Heindel 1994; Rapp *et al.* 1997). Therefore, if higher levels of apoE have a detrimental effect on cognitive function, it is feasible that the clusters of apoE observed here could contribute to the age-related decline in cognition.

As apoE has an integral role in the catabolism of amyloid within the brain, apoE might be increased in the elderly rhesus monkey brain for clearance of the age-related increase in A β (Cork *et al.* 1990; Uno *et al.* 1996; Sani *et al.* 2003). Similar to previous studies, we demonstrate that apoE clusters are co-localized with A β deposits in aged rhesus macaques (Mufson *et al.* 1994; Hartig *et al.* 1997; Sani *et al.* 2003). Furthermore, all apoE clusters were A β -positive, but not all A β -clusters were apoE-positive,

which has been previously described (Nishiyama *et al.* 1997). Increased A β deposits are found in humans who express apoE ϵ 4, a risk factor for developing AD (Poirier 1994; Poirier 2000). Rhesus macaques only have one isoform of apoE, the ϵ 4 allele (Gearing *et al.* 1994; Mufson *et al.* 1994; Poduri *et al.* 1994), but do not develop severe cognitive deficits associated with AD. One potential reason for the lack of behavioral deficits as observed with AD is that the apoE ϵ 4 allele in the rhesus macaque does not have arginine at position 61, which is responsible for the domain interaction observed with human apoE ϵ 4 (Morelli *et al.* 1996; Hatters *et al.* 2006). Thus, the rhesus macaque apoE ϵ 4 allele is more structurally similar to the human apoE ϵ 3 allele.

In rodent studies, GFAP has been reported to increase with age (Lindsey *et al.* 1979; Bjorklund *et al.* 1985; Kohama *et al.* 1995). Similarly, GFAP synthesis, protein, and degraded products increase with age in subcortical white matter of a nonhuman primate (Sloane *et al.* 2000). Here, we report an increase in GFAP immunoreactivity, without an increase in the number of cells expressing GFAP. Thus, the apparent increase in GFAP immunoreactivity observed in this study is most likely due to the increase in cell size and number of processes expressing GFAP, corroborating rodent studies (Landfield *et al.* 1977; Lindsey *et al.* 1979; Garcia-Segura *et al.*, 1994a, b; Garcia-Segura *et al.* 1994a, b; Berciano *et al.* 1995; Garcia-Segura *et al.* 1996; Struble *et al.* 2006). Moreover, other nonhuman primate studies demonstrate that the cytoarchitecture of glial cells change with age but not the number of glial cells (Peters and Sethares 2002; Sandell and Peters 2002). Therefore, the age-related increase of GFAP suggests an increase in plasticity of GFAP-expressing astrocytes, or an increase in GFAP within astrocytes previously expressing low levels of GFAP (Sloane *et al.* 2000).

Notably, the pattern of GFAP immunoreactivity was different from that of apoE. Although GFAP and apoE immunoreactivity show little colocalization at all ages, apoE clusters found in the elderly animals contain groups of GFAP cells that appear to infiltrate the apoE clusters (Fig. 9c). Due to the location of GFAP cells and the apoE clusters, it is possible GFAP contributes to the formation or function of these clusters. Thus, the increase in GFAP and apoE might be related, and the increase in GFAP could be part of a compensatory mechanism for the age-related increase in apoE.

We report an age-related increase of area occupied by immunofluorescence of MAP-2, apoE, and GFAP, as well as a decrease of SYN. Although we did not count synapses, synaptic vessels, or connections, Western blot analyses support our immunohistochemistry findings. However, the sensitivity of the two techniques is slightly different. For instance, Western blot analysis did not detect the age-related changes in MAP-2 between adult and middle-aged animals that were observed in the immunohistochemical analysis. As expected, these data indicate that immunohistochemistry is more sensitive to detect relatively smaller age-related changes in MAP-2. Similarly, Western blot analysis of GFAP only showed a trend towards a

change in the PFC. Thus, the age-related change in GFAP in the PFC was not robust enough to be identified by Western blot analysis. The lack of a robust increase in GFAP levels as measured by Western blot supports the previous discussion of the apparent increase in GFAP processes and the potential of cells expressing low levels of GFAP increasing their GFAP expression over time. Furthermore, it is possible that the number of MAP-2 synapses increase and the number of SYN synapses or synaptic vesicles decrease. Interestingly, a clear difference in structural immunoreactivity in apoE between the adult and middle-aged animals versus the aged was observed.

In summary, age-related changes are observed in MAP-2, SYN, apoE, and GFAP immunoreactivity and protein levels as assessed by Western blot analysis in the rhesus macaque. These observations are more similar to the changes reported in aging human studies than rodent studies. As such, these results further support the rhesus macaque as a valuable translational model of human aging and a useful tool in understanding the biology of the aging brain.

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