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Mitochondrial base excision repair in mouse synaptosomes during normal aging and in a model of Alzheimer's disease

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Abstract

Brain aging is associated with synaptic decline and cognitive impairment. Increased levels of oxidative DNA base damage and accumulation of mitochondrial DNA (mtDNA) mutations or deletions lead to mitochondrial dysfunction, playing an important role in the aging process and the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD). In mitochondria, base excision repair (BER) is the main DNA repair pathway for base modifications such as deamination and oxidation, and constitutes an important mechanism to avoid accumulation of mtDNA mutations. Synaptic function is highly dependent on mitochondria, and in the current study we have investigated BER in synaptosomes of mouse brain during normal aging and in an AD model. Synaptosomes are isolated synapses in membranous structures produced by subcellular fractionation of brain tissue. They include the whole presynaptic terminal as well as portions of the postsynaptic terminal. Synaptosomes contain the molecular machinery necessary for uptake, storage, and release of neurotransmitters, including synaptic vesicles and mitochondria. BER activities were measured in synaptosomal fractions from young and old mice and from pre-symptomatic and symptomatic AD mice harboring mutated APP, Tau and PS1 (3xTgAD). During normal aging, a reduction in the BER capacity was observed in the synaptosomal fraction, which was associated with a decrease in the level of BER proteins. However, we did not observe changes between the synaptosomal BER activities of pre-symptomatic and symptomatic AD mice. Our findings suggest that the age-related reduction in BER capacity in the synaptosomal fraction might contribute to mitochondrial and synaptic dysfunction during aging. The development of AD-like pathology in the 3xTgAD mouse model was, however, not associated with deficiencies of the BER mechanisms in the synaptosomal fraction when the whole brain was analyzed.

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Keywords

DNA repair; Mitochondria; Synaptic dysfunction; Neurodegeneration

1. Introduction

Brain aging is characterized by cognitive and behavioral decline. Decrease in spine densities (Dickstein et al., 2007), alterations in signaling pathways (Dröge and Schipper, 2007; Foster, 2007) and neurotransmitter systems (Mora et al., 2007) occur with normal aging, favoring general neuronal dysfunction. These changes are described to occur mainly in cerebral cortex and hippocampus (Mora et al., 2007), but also in cerebellum (Huang et al., 2006; Jernigan et al., 2001). Moreover, these changes are exacerbated in specific brain regions when neurodegenerative disorders such as Alzheimer's disease (AD) develop during aging (Dickstein et al., 2007; Mattson, 2004).

Mitochondrial DNA (mtDNA) mutations and mitochondrial dysfunction are thought to play an important role in the aging process (Barja, 2004; Cantuti-Castelvetri et al., 2005; Kujoth et al., 2007); and increased levels of oxidative modifications and mutations in mtDNA occur in the brain during normal aging (Beal, 2005; Melov, 2004; Vermulst et al., 2007) and in AD (Gabbita et al., 1998; Morocz et al., 2002). Base excision repair (BER) is the primary DNA repair pathway for small DNA modifications caused by alkylation, deamination or oxidation in nuclei and mitochondria and it has been described to play a major role in the development and maintenance of the central nervous system (CNS) (Weissman et al., 2007a). The BER pathway includes four distinct steps (Bohr, 2002). First, DNA glycosylases are responsible for recognition and removal of the modified bases, rendering an abasic site, which is then processed by AP endonuclease (APE). Repair can then proceed through one of two subpathways: short- or long-patch BER, both taking place in the nucleus and in the mitochondrion (Akbari et al., 2008; Bohr, 2002; Liu et al., 2008). The short-patch BER involves the incorporation of a single nucleotide into the gap by DNA polymerase followed by strand ligation by DNA ligase, while long-patch BER involves incorporation of several nucleotides, typically two to seven, followed by cleavage of the resulting 5' flap and ligation. In mitochondria, polymerase gamma (pol γ) is the only polymerase present, being involved both in replication and repair events. Although mitochondria possess an independent BER machinery, the mitochondrial BER components are encoded by nuclear genes (Bohr, 2002).

Increased neuronal mtDNA instability is especially important when occurring in mitochondria located at the synaptic terminals, which are characterized by high-energy requirements. Mitochondria at that location provide energy for critical processes such as exocytosis/ endocytosis events of synaptic vesicles or the preservation of ionic strength. Thus, these mitochondria play a central role in synaptic activity and therefore in the proper function of the CNS (Brown et al., 2006; Foster, 2007; Ly and Verstreken, 2006). Various investigations have reported important biochemical differences between synaptic mitochondria and those located in the neuronal soma (Borras et al., 2003; Brown et al., 2006; Lai et al., 1977). They also behave differently when physiological conditions change (Martinez et al 2009), suggesting that these mitochondrial subpopulations might also have different vulnerability to aging. Various studies suggest that synaptic impairment is associated with mitochondrial dysfunction during aging and as an early event in AD (Fontán-Lozano et al., 2008; Mattson et al., 1998; Selkoe, 2002), but it is not known whether mtDNA repair mechanisms play a significant role in this process. The first reports describing the presence of a DNA repair protein, pol γ , in synaptic brain mitochondria were published in the seventies (Hübscher et al., 1977; Hübscher et al., 1979). However, those

investigations only focused on the role of pol γ in mtDNA replication, since, at that time, it was believed that mitochondria lacked the enzymes necessary for DNA repair (Clayton et al., 1974). More recently, Cortina et al (2005) have described the regulation of pol γ and 8-oxoguanine-DNA glycosylase levels in synaptic mitochondria in photoreceptors after retinal damage induced by light. Yet, the DNA repair mechanisms in the synaptic fraction of the brain have not previously been investigated.

In this study, we investigated BER activities of the synaptic fraction from whole mouse brains and whether they may play a significant role in the synaptic loss associated with aging and AD. We approached this study by purifying synaptosomes from normal aged mice and from an AD mouse model, 3xTgAD mice carrying $PS1_{M146V}$, APP_{Swe} and Tau_{P301L} transgenes (Oddo et al., 2003a). Synaptosomes have been widely used in neurobiology as an approach to synaptic research and have been extensively characterized (Borras et al., 2003; Brown et al., 2006; Hübscher et al., 1979; Lai et al., 1977; Mattson et al., 1998; Schimpf et al., 2005). Our findings showed that a significant age-related decline of BER capacity occur specifically at the synapses, partly due to a reduction in the level of BER proteins in that fraction. However, the development of synaptic impairment that is observed in 3xTgAD mice is not reflected in a decline in the BER capacity at the synapses, at least when the whole brain is investigated.

2. Materials and methods

2.1 Animals

Male 129B6F1 mice were bred and fed at the University of Aarhus. Animals were sacrificed by decapitation at age 5 weeks (young), 5 months (adult) and 23 months (old) and whole brains were rapidly removed. Synaptosomal and free brain mitochondrial (FBM) fractions were purified and used to investigate the role of BER in synaptic loss during normal aging. On the other hand, the role of BER in synaptic impairment during AD was investigated by using male 3xTgAD mice carrying $PS1_{M146V}$, APP_{Swe} , and Tau_{P301L} transgenes (Oddo et al., 2003a). Male C57BL/6 age-matched animals served as controls. The 3xTgAD mice were bred to homozygosity and backcrossed to C57BL/6 mice for 7 generations. In 3xTgAD animals, A β accumulation is first detectable in cortical brain regions, primarily frontal cortex and hippocampus at 6 months of age (Oddo et al., 2003b) along with impairment of synaptic transmission (Oddo et al., 2003a). Since the purpose of the investigation was to study the association between the appearance of A β pathology, synaptic failure and BER, we compared 3 month-old, presymptomatic mice to 12 month-old animals in which A β pathology is clearly evident. Animals were sacrificed by decapitation and brains were immediately removed for synaptosomal purification.

All mice were fed regular Purina animal chow *ad libitum* and kept in a 12 h light /dark cycle. All experiments were approved by the NIA IACUC and were performed in accordance with the "Guidelines for the use and care of laboratory animals (NIH Publications 85-23).

2.2 Purification of synaptosomal and free brain mitochondrial fractions

Synaptosomes were isolated as previously described elsewhere (Lai and Clark, 1979). This purification protocol of synaptosomes allows simultaneous isolation of non-synaptic mitochondria, also called free brain mitochondria (FBM). All steps were carried out at 4°C. Briefly, whole brains were gently homogenized in STE buffer (32 mM Sucrose, 10 mM TrisHCl, 1 mM EDTA; pH 7.4) with a glass-glass homogeniser. All steps were performed in the presence of protease inhibitors (0.15 mM spermine, 0.75 mM spermidine, 1 mM PMSF, 5 mM DTT, 1:1000 protease inhibitor cocktail set III (Calbiochem)). The nuclear fraction

was discarded by centrifugation at 1,300 g for 10 minutes twice. The fraction containing synaptosomes and FBM was spun down by centrifugation of the resultant supernatant at 17,000 g for 10 min. The resulting supernatant (representing the crude cytosolic fraction) was aliquoted and stored at -80°C until use for further analysis. The pellet was resuspended in STE buffer and layered on a discontinuous Ficoll gradient 7.5–12 %. After centrifugation at 99,000 g for 40 min, the synaptosomal fraction was obtained at the 7.5–12 % interface, while the pellet comprised the FBM fraction. Final synaptosomal and FBM fractions were obtained after washing with STE buffer and centrifugation at 18,500 g and 9,800 g for 10 min, respectively. Samples were resuspended in 20 mM HEPES pH 7.4, 1 mM EDTA, 2 mM DTT, 5% glycerol, aliquoted and stored at -80°C until use. Protein concentration of the different preparations was determined by the Lowry method (Lowry et al., 1951).

2.3 Transmission electron microscopy

Synaptosomal fractions were analyzed by transmission electron microscopy (TEM). The samples were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer with 3 mM CaCl_2 for 1 hour at pH 7.2–7.4 at room temperature. After rinsing the samples three times for 5 minutes in 0.1 M cacodylate and 3 mM CaCl_2 , they were post fixed with 2 % OsO_4 in 0.1 M cacodylate and 3 mM CaCl_2 for 1 hour at 4°C . Samples were washed with dH_2O and treated with 2% Uranyl Acetate for 30 minutes in the dark. Dehydration was performed with series of ethanol: 50%, 70%, and 90% for 5 minutes each and final dehydration of 100% ethanol, 3 times for 5 minutes each. Afterwards samples were dehydrated 2 times for 5 minutes in Propylene Oxide and infiltrated with 1:1 ratio of Propylene Oxide to Epon with catalyst for 1 hr. Samples were rotated overnight in 100% Epon with catalyst. The following day, Epon with catalyst was changed 3 times allowing 2 hours in between each change and samples were placed in an oven at 60°C for 2 days. Finally, the samples were analyzed by TEM.

2.4 Western blotting

Samples (50 μg protein) were separated on 4–12% NuPAGE Novex® Bis-Tris gels (Invitrogen) and transferred to PVDF membranes (Invitrogen). In order to confirm the absence of nuclear contamination in the preparations, the nuclear protein lamin B was assayed by using a goat polyclonal antibody (1:1000; Santa Cruz Biotechnology). Mitochondrial content was assayed by detection of the outer mitochondrial protein VDAC1 with a rabbit polyclonal antibody (1:500; Abcam). BER proteins APE and UNG1 were detected by using mouse monoclonal (1:2000; Novus biologicals) and rabbit polyclonal (1:1000; Santa Cruz Biotechnology) antibodies, respectively. Kinesin rabbit polyclonal antibody (1:2000, Abcam;) and Actin goat polyclonal antibody (1:5000, Santa Cruz biotechnology) were assayed in the crude cytosolic fraction. Secondary anti-goat, anti-rabbit or anti-mouse antibodies were applied at 1:3000 – 1:5000. Membranes were visualized using ECL plus® (GE Healthcare, Amersham).

2.5 Oligonucleotides

Oligonucleotides were purchased from DNA Technology (Aarhus, Denmark) and from the Midland Certified Reagent Company (Midland, TX, USA) and were 5'-end-labeled using T4 polynucleotide kinase (PNK) and $\gamma\text{-}^{32}\text{P}\text{-ATP}$. Mixtures of 100 μg oligonucleotide containing the DNA lesion or an unmodified base (Table 1), 20 units of T4 PNK (Fermentas), PNK forward buffer A (Fermentas) and 333 μCi $\gamma\text{-}^{32}\text{P}\text{-ATP}$ were incubated for 90 min at 37°C , and then stopped with EDTA to a final concentration of 0.05M. The unincorporated $\gamma\text{-}^{32}\text{P}\text{-ATP}$ was removed by binding to G25 Microspin columns (GE Healthcare, Amersham). Duplexing of the 5'-end-labeled oligonucleotide with the corresponding complementary strand was carried out by heating to 90°C followed by gradual cooling to room temperature.

2.6 Base excision repair synthesis assays

Repair synthesis was quantified by using a non-labeled 30 base-pair hairpin loop containing deoxyuracil (Table 1), essentially as described in Nyaga and Bohr (2002). Reactions contained 40 mM HEPES pH 7.8, 0.1 mM EDTA, 5 mM MgCl₂, 0.2 mg/ml BSA, 50 mM KCl, 1 mM DTT, 40 mM phosphocreatine, 100 µg/ml phosphocreatine kinase, 2 mM ATP, 20 µM dNTPs, 4 µCi ³²P-dCTP, 3% glycerol, and 100 ng double-stranded oligonucleotide, in 50 µl. 30 µg and 50 µg of synaptosomal and FBM fractions, respectively, were added, and reaction mixtures were incubated at 37°C for 3 hours. Reactions were terminated by adding 12.5 µg of proteinase K (PNK) and 2.5 µl of 10% SDS and incubating at 55°C for 30 min. DNA was precipitated by addition of 1 µg glycogen, 4 µl of 11 M ammonium acetate, 60 µl of ethanol and overnight incubation at -20°C. DNA was centrifuged, dried and resuspended in 20 µl of formamide loading dye (80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol FF, and 1 mg/ml Bromophenol Blue) and loaded on a 20% denaturing polyacrylamide gel. The incorporated radioactively labeled dCTP was visualized using a Personal Molecular Imager™ and quantified using Quantity One software (Bio-Rad).

2.7 DNA glycosylase activities

The activity of various DNA glycosylases in synaptosomal and FBM fractions was determined *in vitro* by incision assays for uracil, 5-OH-cytosine (5-OHC) and 8-oxo-guanine (8-oxoG) as previously described (Souza-Pinto et al., 1999). 10 µg (uracil) or 20 µg (5-OHC and 8-oxoG) of synaptosomal extracts were incubated with 100 fmol of the corresponding duplexed ³²P-labelled oligonucleotide (Table 1) in a 20 µl reaction. Reactions with uracil-containing oligonucleotide included 20 mM HEPES pH 7.6, 5 mM EDTA, 75 mM KCl, 1 mM MgCl₂, 5 % glycerol, 5 mM DTT and 0.1 mg/ml BSA. Reactions with 8-oxoG or 5-OHC oligonucleotides were slightly different, containing 40 mM Hepes-KOH pH 7.6, 5mM EDTA, 75 mM NaCl, 1 mM DTT, 10% glycerol and 70 mM Hepes-KOH pH 7.6, 5mM EDTA, 75 mM NaCl, 1 mM DTT, 10% glycerol.

The amount of FBM extracts added to the reactions were the same as those of synaptosomal extracts except for reactions with 8-oxoG-containing oligonucleotide, where 60 µg of FBM extract was used. After incubating for one hour (uracil), 6 hours (5-OHC) or 18 hours (8-oxoG), reactions were stopped by addition of 0.5 % SDS and 0.25 µg/µl proteinase K, followed by 30 minutes incubation at 55°C. Samples incubated with uracil-containing oligonucleotide were mixed with 20 µl of formamide dye. DNA from the 5-OHC- and 8-oxoG- containing reactions was ethanol-precipitated by the addition of 0.5 µg of glycogen, 2 µl of 11 M ammonium acetate and 63 µl of ethanol, pelleted, dried and resuspended in formamide loading dye. All samples were loaded on a 20% denaturing polyacrylamide gel and the radioactively labeled DNA was visualized using a Personal Molecular Imager™ and quantified using Quantity One software (Bio-Rad). Incision activities were calculated as the amount of radioactivity in the band corresponding to the damage-specific cleavage product divided by the total radioactivity in the whole lane.

2.8 AP endonuclease assays

APE activity in synaptosomal and FBM fractions was assayed using an oligonucleotide containing the abasic site analog tetrahydrofuran (THF; Table 1). When extracts from young and aged mice were investigated, reactions contained 20 mM HEPES pH 7.6, 5 mM EDTA, 75 mM KCl, 1 mM MgCl₂, 5 % glycerol, 5 mM DTT and 0.1 mg/ml BSA. 100 fmol of double-stranded THF-containing oligonucleotide substrate and 200 ng (synaptosomal fraction) or 500 ng (FBM fraction) of extract were added to a final reaction volume of 20 µl. Reaction mixtures were incubated at 37°C for 30 minutes and the reactions were terminated by addition of 0.5 % SDS and 0.25 µg/µl proteinase K, followed by 30 minutes incubation at 55°C. The procedure varied slightly when 3xTgAD mice were investigated. Reactions

contained 25 mM HEPES pH 7.4, 25 mM KCl, 5 mM MgCl₂, 10 % glycerol and 0.1 mg/ml BSA. One hundred fmol of double-strand THF-containing oligonucleotide substrate and 50 ng (synaptosomal fraction) or 100 ng (FBM fraction) of extract were added to a final reaction volume of 20 μ l. Reaction mixtures were incubated at 37°C for 10 minutes and were terminated by addition of formamide loading dye and heating at 90°C for 10 minutes. Samples were visualized and analysed as described for the measurement of DNA glycosylase activities.

2.9 Gap-filling assay

Polymerase single nucleotide gap-filling activity was measured using a non-labeled 34-mer duplex oligonucleotide containing a single gap at position 16 (Table 1). Reactions (10 μ l) contained 40 mM Hepes KOH (pH 7.6), 0.1 mM EDTA, 50 mM KCl, 0.2 mg/ml BSA, 1 mM DTT, 5 mM MgCl₂, 3 % glycerol, 1 μ g poly dI-dC, 4 μ M dCTP (Roche Applied Sciences, Indianapolis, IN, USA) and 2 μ Ci of ³²P-dCTP (GE Healthcare Corp. Piscataway, NJ, USA). 1 μ g of synaptosomal extracts were incubated for 2 hours at 37°C, while 0.5 μ g of FBM extracts were incubated for 1 hour at 37°C. All reactions were terminated by the addition of formamide loading dye and heating at 90°C for 10 minutes. Samples were resolved and visualized as described above for 8-oxoG and 5-OHC incision assays.

2.10 Statistical analysis

All data are reported as mean $\pm$ standard deviation (SD) of at least 3 independent synaptosomal preparations, each purified from one mouse brain. For each independent sample, all assays were performed at least in duplicate. Comparisons of groups were analyzed statistically by Student's t test. The minimum level of statistical significance was set at $p \leq 0.05$ for all analyses.

3. Results

The main aim of the current study was to investigate the DNA repair capacity of the synaptosomal fraction of the mouse brain and analyze whether putative changes in DNA repair capacity with aging might contribute to increased mtDNA instability and synaptic failure. As described in materials and methods, synaptosomes were isolated by differential and density-gradient centrifugation. In order to confirm the presence of synaptosomal structures and mitochondrial proteins, synaptosomal fractions were analysed by electron microscopy imaging as well as by western blotting (Figure 1). Electron microscopy analysis confirmed the presence of synaptosomal structures, with evident mitochondria and synaptic vesicles within membranous structures. These results were similar to those previously reported by others (Hübscher et al., 1977; Rendon and Masmoudi, 1985; Schrimpf et al., 2005). Mitochondrial content was tested by western blotting against the mitochondrial outer membrane protein VDAC1 (Figure 1), and the lack of nuclear contamination was confirmed by the absence of Lamin B in synaptosomal fractions (Figure 1) and free brain mitochondria (data not shown).

DNA repair capacity in brain synapses has not previously been investigated in detail. Since mitochondria have an active BER pathway, DNA repair activities were expected to be detected in synaptosomal fractions. The ability of synaptosomes to remove DNA damage, to process and fill the resultant gap, and to religate the DNA strand (total BER product) was investigated by analysis of the incorporation of ³²P-dCTP into a non-labeled uracil-containing oligonucleotide. BER activity was detected in synaptosomal fractions (Figure 2A) and was specific for the lesion-containing oligonucleotide since no incorporation was observed in undamaged oligonucleotides. Likewise, individual steps of the BER pathway were analysed. Recognition and cleavage of the lesions by DNA glycosylases were

investigated using uracil- and 8oxodG-containing oligonucleotides (Figure 2B), while a THF-containing oligonucleotide was used in order to measure the APE activity (Figure 2C). Optimal extract concentrations for each assay were determined to ensure that repair capacity was not saturated under those experimental conditions.

3.1 DNA repair capacity in young mice is higher in synaptosomes than in free brain mitochondria

Since synaptic mitochondria are subjected to a differential cellular environment, our first interest was to investigate whether the DNA repair capacity at synapses differ from that in free brain mitochondria. While the free brain mitochondrial fraction is mitochondria-enriched, synaptosomes contain not only mitochondria but also cellular components enclosed within the membranous structure. In accordance with this notion, the total mitochondrial content, estimated as the level of the mitochondrial outer membrane protein VDAC1, in the synaptosomal fraction was significantly lower ($p \leq 0.001$) than in the free brain mitochondrial fraction (Figure 3A). The synaptosomal fraction also showed a significantly lower ($p \leq 0.01$) cytochrome c oxidase (COX) activity than the free brain mitochondrial fraction (Figure 3A). Since mitochondrial content differed between the two fractions, DNA repair activities were normalized to COX activity or mitochondrial protein content, observing the same result in both cases. Synaptic mitochondria showed significantly higher capacity of recognition and removal of uracil (Figure 3B) and 8-oxoG (Figure 3C) in double stranded oligonucleotides than free brain mitochondria ($p \leq 0.05$). Increased activities in the synaptosomal fraction were also observed when analysing APE ($p = 0.02$) (Figure 3D) and the whole uracil-initiated BER pathway ($p = 0.02$) (Figure 3E). UNG1, APE and the uracil-initiated BER activities were 40% higher in synaptosomes than in free brain mitochondria.

3.2 Base excision repair activity declines with aging in synaptosomes but not in free brain mitochondria

We investigated whether impairment in DNA repair in synaptosomes during aging might contribute to the age-related decline in synaptic function. We also analysed the DNA repair mechanisms in free brain mitochondria, in order to explore whether putative age-related changes were taking place in general or in a particular mitochondrial subpopulation.

Synaptosomes and free brain mitochondria from mice of three age groups were purified and the total BER capacity was measured. As shown in figure 4A, a significant progressive decline in the total BER activity occurred with aging in the synaptosomal fraction ($p \leq 0.01$). Such decline was specific to that particular fraction, since an increase in the total BER activity took place in the free brain mitochondrial preparations (Figure 4B). However, this increase was not statistically significant ($p = 0.10$). These results indicate that the BER pathway is distinctly affected during aging in synaptic mitochondria. To further investigate the age-related decline in the synaptosomal BER, we analysed the first two steps of the pathway: the recognition and cleavage of the lesion by UNG1 and the processing of the abasic site by APE. Both UNG1 (Figure 5A) and APE (Figure 5B) activities were significantly reduced in synaptosomal fractions from old mice ($p \leq 0.05$) compared to young ones. However, while APE activity was more gradually reduced, UNG1 activity was significantly reduced at the age of 5 months ($p < 0.001$) and remained constant during adulthood. The reductions in APE and UNG1 activities were not due to lower mitochondrial content among the fractions purified from different age groups, as assayed by the levels of the mitochondrial protein VDAC1 (Figure 5C). Interestingly, when those activities were analyzed in free brain mitochondria, no significant changes ($p = 0.07$) were observed for UNG activity (Fig 5D), while a significant increase was observed for APE (Figure 5E).

Thus, similarly to the total BER activity, the decline in UNG1 and APE activities occurred specifically in the synaptosomal fraction.

3.3 Lower DNA repair activity with aging in synaptosomes is associated with reduction in the levels of DNA repair enzymes and reduced mitochondrial axonal transport

In order to investigate the mechanisms underlying the reduced BER activities in synaptosomes from old mice, the level of UNG1 and APE proteins were determined. Indeed, the protein levels of both UNG1 (Figure 6A) and APE (Figure 6B) were progressively and significantly reduced during lifespan ($p \leq 0.05$). Moreover, the magnitude of the reduction in protein levels between the young and old groups corresponded to the reduction in activity of both enzymes, 40% in UNG1 and 20% in APE, suggesting that the age-related decrease of BER activities in the synaptosomal fraction was primarily due to the decline in protein level.

We also investigated whether the reduction of the BER protein levels in the synaptosomal fraction was related to impairment in axonal transport from the neuronal soma to the synapses. A significant reduction in the levels of the main motor protein involved in mitochondrial axonal transport, kinesin, was observed during lifespan ($p \leq 0.05$) (Figure 6C).

3.4 Synaptosomal BER activities are not affected in a mouse model of Alzheimer's disease

Alzheimer's disease is a severe neurodegenerative disease associated with a remarkable loss of synapses and impairment of cognitive function. Previous investigations in our research group have shown significant BER deficiencies in brains of sporadic AD patients due to limited DNA glycosylase and polymerase β activities (Weissman et al., 2007b). However, despite the deficiencies in BER activities found in human AD brains, no changes in BER activities were found in different brain regions of AD mouse models using whole tissue extracts (Weissman et al., 2009). Synaptic dysfunction and failure are processes that occur early in AD (Knobloch and Mansuy, 2008; Mattson et al., 1998), and in the current study we investigated whether those same activities might be reduced particularly in the synaptosomal fraction in one of those AD mouse models, the 3xTgAD. DNA damage processing by DNA glycosylases and APE activity as well as DNA synthesis capacity by DNA polymerase were studied. No changes were observed between WT and 3xTgAD mice of any age group, or between pre-symptomatic and symptomatic 3xTgAD mice in any of the activities analysed either in the synaptosomal fraction (Figure 7) or in the free brain mitochondrial fraction (data not shown), suggesting that the impairment in synaptic function that has been reported to occur in 3xTgAD mice after 6 months of age (Oddo et al., 2003a) is not associated with deficiencies in synaptosomal BER when whole brain is studied.

4. Discussion

The aim of the current study was to determine whether synaptic impairment during normal aging and in Alzheimer's disease is associated with a decline in mitochondrial DNA repair at the synapses. Our results indicate that during normal aging a significant mtBER decline occurs specifically in the synaptosomal fraction, likely contributing to synaptic dysfunction via mitochondrial impairment. However, the synaptic decline previously observed in the AD mouse model 3xTgAD is not associated with changes in the BER capacity of the synaptosomal fraction, at least when whole brains are investigated, as we did in our present work.

Although different studies have investigated age-related changes in DNA repair mechanisms in brain mitochondria (Gredilla et al 2008, Imam et al 2007, Chen et al 2002), no previous studies concerning DNA damage repair have been performed specifically in synaptosomes from brain. Thus, we first characterized the BER activities of the synaptosomal fraction in

young animals. It is well established that BER takes place in mitochondria (Bohr, 2002), and as expected, BER activities were clearly measurable in the synaptosomal fractions. Because DNA repair activities exist both in the nucleus and in mitochondria of mammalian cells, the absence of nuclear contamination in the synaptosomal fractions ensured us that the measured BER activities in these fractions were mitochondrial.

Although the free brain mitochondrial fraction could contain perinuclear mitochondria from neurons as well as mitochondria from glia cells, a recent investigation using a similar method for purification of free brain mitochondria reported that the free brain mitochondrial fraction was predominantly of neuronal origin (Kristian et al., 2006). Our results clearly showed that BER capacity was lower in free brain mitochondria than in the synaptosomal fraction, and constitute the first evidence for the existence of differences between synaptosomal and free brain mitochondrial DNA repair activities. These findings are of great interest for future studies, since they emphasize the notion that synaptosomal mitochondria possess specific DNA repair properties. Different reports have suggested that BER activities are regulated, at least in part, by oxidative stress (Chen et al., 2003; Ogihara et al., 2002; Stuart et al., 2004). In fact, when highly specific methods for mitochondrial reactive oxygen species (ROS) detection have been used to determine mitochondrial free radical generation (Barja, 2002), the rate of mitochondrial ROS production was higher in synaptic than in non-synaptic mitochondria (Borras et al., 2003). Moreover, reactive nitrogen species (RNS) are important sources of DNA damage in synapses due to the role of nitric oxide as intercellular messenger. As a consequence, synaptic mitochondria are highly exposed to DNA damaging agents. Thus, it is likely that the enhanced BER capacity in the synaptosomal fraction is essential in order to prevent DNA instability and preserve mitochondrial function at the synapses.

Interestingly, the heterogeneity between the mitochondrial subpopulations was not restricted to the repair capacity at young age. In agreement with an earlier report from our group (Imam et al., 2006), we observed no significant age-related changes in the total uracil-initiated BER activity in free brain mitochondria. However, we observed a progressive decline from young to old animals in the total uracil-initiated BER capacity in the synaptosomal fraction. A reduced activity in the synaptosomal fraction of old animals was also observed when UNG1 and APE activities were investigated.

Nevertheless, whereas APE activity gradually declined during aging, UNG1 activity had already declined maximally in adult animals and did not change with increased age thereafter. UNG1 has previously been shown to exhibit a unique activity-pattern in brain mitochondria during early stages of life, when developmental changes in mouse brain are still not complete (Gredilla et al., 2008). An important regulator in vertebrate development of the central nervous system is nitric oxide, and hence the mitochondrial levels of RNS are likely to be high during brain maturation. Thus, mtDNA is prone to deoxy-uracil base modifications due to deamination of cytosine by RNS (Reddy et al., 2007). Higher levels of UNG1 during maturation would ensure the proper recognition and removal of this particular base modification in mtDNA. Nevertheless, although the individual mtBER steps may be differently affected at the synapses during aging, the whole BER capacity is reduced during aging in that particular fraction.

Although it is not known whether mtDNA lesions accumulate with aging specifically in synaptic mitochondria, our findings suggest that the reduction in BER activities during aging might contribute to an increase in mutagenic DNA lesion levels in synaptic mitochondria, leading to mitochondrial dysfunction and synaptic impairment. Although further investigation is needed to determine whether such accumulation takes place, it has recently been described that an increase in free radical generation occurs during aging in synaptic

mitochondria (Kilbride et al., 2008). An imbalance between DNA damage and DNA repair in the synaptosomal fraction during aging could be an important factor contributing to synaptic and cognitive age-associated decline.

Different studies have demonstrated that functional brain decline during aging is not due to neuronal loss in main brain regions like cortex and hippocampus (Morrison and Hof, 2002; West et al., 1994; West et al., 2004), and it has been suggested that the degeneration and loss of dendrites and synapses are key factors in functional brain decline and neuronal aging, with mitochondria as main determinants (Dickstein et al., 2007; Selkoe, 2002). In accordance with these studies, our results show that synaptic mitochondria may be more susceptible to age-related impairment than those located in other neuronal compartments due to the mtBER decline that takes place specifically at the synapses with aging. Increased mtDNA instability could compromise synaptic mitochondrial function, and hence synaptic function, contributing to neuronal aging.

In order to investigate the mechanisms underlying the mtBER decline at the synapses, we analyzed the protein levels of the BER enzymes APE and UNG1. In both cases, we observed a gradual decline in protein levels during the lifespan. The main mechanism responsible for the reduced APE activity with aging in the synaptosomal fraction seemed to be a decrease in the protein level. The decrease in APE protein level in the synaptosomal fraction corresponded to the reduction in APE activity among age-groups in this fraction. Interestingly, the remarkable drop that UNG1 activity exhibited from the young to the adult group was not due to a corresponding decrease in UNG1 protein levels, suggesting that the tuning of the UNG1 activity was not exclusively dependent on protein level, but also on other mechanisms. These could for example include post-translational modifications since UNG1 has been shown to contain various potential protein kinase C phosphorylation sites (Chatterjee and Singh, 2001) and Hagen et al. (2008) recently reported that the catalytic activity and protein interaction of the nuclear isoform of UNG is regulated by phosphorylation during the cell cycle of human HeLa cells.

The gradual decrease in BER enzymes was not a consequence of a general decline in mitochondrial BER protein levels in the brain, since we did not find significant changes in the level of UNG1 protein with aging when studying the free brain mitochondrial fraction (data not shown). We hypothesized that the reduction in BER protein levels in synaptic mitochondria was most likely related to the characteristic location of synaptic mitochondria and their dependence on axonal trafficking. The mitochondrial BER pathway at the synapse is dependent upon anterograde axonal transport, since mitochondrial biogenesis and synthesis of nuclear encoded mitochondrial proteins, including those involved in mitochondrial BER, take place primarily in the neuronal cell body. Moreover, degradation of synaptic mitochondria is dependent on retrograde axonal transport due to the lack of lysosomes at synaptic ends (Hollenbeck, 1993; Tatsua and Langer, 2008). In agreement with previous reports (Cross et al., 2008; Hollenbeck and Saxton, 2005; Kimura et al., 2007), we did observe a significant age-related reduction in the level of the cytosolic protein kinesin, the main motor protein involved in anterograde mitochondrial axonal transport. Moreover, different studies have reported that retrograde axonal transport proteins are also affected during aging, as well as in neurodegenerative diseases, contributing to impaired retrograde transport of mitochondria and other organelles such as endosomes (Stokin and Goldstein, 2006; Kimura et al., 2009; Shi et al., 2010). The reduction in both anterograde and retrograde transport through the axon might affect the turnover of synaptic mitochondria, promoting the accumulation of oxidized molecules. It is likely that the reduction in BER protein levels in the synaptosomal fraction of old mice is a consequence of the degradation processes by the intramitochondrial proteolytic pathway (Bota and Davies, 2001). The main

consequence would be the reduction in BER capacity in synaptic mitochondria, which would promote the accumulation of mutagenic lesions and hence DNA instability.

In contrast to normal aging, Alzheimer's disease is accompanied by neuronal loss. Moreover, synaptic loss is currently the best pathologic correlate of cognitive decline, and synaptic dysfunction occurs before synapses and neurons are lost (Dickstein et al 2007, Selkoe, 2002). In tissue extracts from specific brain regions of post-mortem samples of AD patients and age-matched controls we found that a significant reduction in BER capacity occurs in AD (Weissman et al., 2007b). While AD research in human brain samples is limited by available autopsy samples, mouse models provide an important tool in AD research from early stages of the disease throughout its progression. However, in contrast to the results in human AD brains, we have recently reported that the development of AD-like pathology in two AD mouse models is not associated with deficiencies in BER activity in tissue extracts from various brain regions (Weissman et al., 2009). The strength of AD mouse models in AD research might be limited if the mechanisms involved in the progression of the pathology differ between species.

Considering that synaptic loss is a critical feature in AD, both in humans and mouse models, we investigated whether specific changes in BER capacity took place in synaptosomal fractions of the 3xTgAD mouse model. A β accumulation and synaptic failure are detectable at six months of age in 3xTgAD mice (Oddo et al., 2003a, Oddo et al., 2003b). Therefore, in order to detect any linkage between BER and the A β -related pathology at the synapses, mice were sacrificed at 3 months (presymptomatic) and 12 months (symptomatic). However, we did not observe any changes in the BER activities either in the synaptosomal or in the free brain mitochondrial fraction. The lack of changes in either fraction might be due to the fact that, in contrast to humans, 3xTgAD mice do not display neuronal degeneration, despite the accumulation of plaques and tangles. Nevertheless, since this study was performed in synaptosomal fractions purified from whole brains, we cannot disregard that a decline in BER capacity in the synaptosomal fraction occur in specific brain regions with vast accumulation of A β and elevated synaptic degeneration, such as hippocampus or cortex (Oddo et al., 2003a, Oddo et al., 2003b).

In conclusion, this investigation demonstrates that a significant decline in the BER capacity during normal aging takes place specifically at the synapses of mouse brain, mainly due to a decrease in the level of BER proteins. Our findings suggest that such decrease in protein level is likely to be associated with the age-related reduction in axonal transport that could affect the turnover of mitochondrial components, likely promoting the degradation of modified BER proteins. Defective synaptic BER may play a critical role in the rise of mtDNA instability and synaptic mitochondrial dysfunction with aging, and may contribute to the synaptic impairment and the cognitive decline reported during normal aging

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Figure 1. Synaptosomal fractions contain significant amounts of mitochondria and are free of nuclear lamin B

TOP: Pictures are representative electron microscopy images of synaptosomal fractions (M: mitochondria). BOTTOM: Presence of mitochondrial content in the synaptosomal fractions was assayed by western blot detection of the mitochondrial protein VDAC1; Lamin B was assayed to confirm absence of nuclear contamination in the different samples; Lanes 1–4: Different synaptosomal fractions; Lane 5: nuclear fraction.

Figure 2. DNA repair capacity in the synaptosomal fraction

(A) Uracil-initiated BER, (B) OGG1 and UNG1 glycosylase activities, and (C) APE activity were assayed by incubating double stranded oligonucleotides containing a specific lesion with increasing concentrations of synaptosomal extracts. Top panels show representative gels; Co: undamaged oligonucleotide; No prot: no protein; S: substrate; P: product. Graphics represent mean $\pm$ S.D. of duplicate measurements from 3 independent samples.

Figure 3. The synaptosomal fraction shows higher DNA repair activities than the free brain mitochondrial fraction

(A) Quantification of mitochondrial content quantified by western blotting against VDAC1 (top) and cytochrome c oxidase activity (COX) (bottom) in the synaptosomal fraction (SYNP) and in the free brain mitochondrial fraction (FBM); bands on the representative WB correspond to independent extracts. (B) UNG1, (C) OGG1, (D) APE and (E) uracil-initiated BER activities were normalized with mitochondrial COX activity. Bars represent mean $\pm$ S.D. of duplicate measurements from 3–5 independent samples. * Denotes statistical significance between groups: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Figure 4. Uracil-initiated BER decreases specifically in the synaptosomal fraction during aging
 Total BER activity in different age groups measured as incorporation of ^{32}P -dCTP in a uracil-containing oligonucleotide in (A) synaptosomal and (B) free brain mitochondrial fractions of young (Y; 5 weeks old) adult (A; 5 months old) and old (O; 23 months old) animals. Top panels show representative gels. Graphics show quantification of the total BER product. Bars represent mean $\pm$ S.D. of duplicate measurements from 3–4 independent samples. (a) Denotes significant differences versus young group; (b) denotes significant differences versus adult group: * $p \leq 0.05$; ** $p \leq 0.01$

Figure 5. DNA repair activities are reduced in the synaptosomal fraction during aging (A, D) UNG1 and (B, E) APE activities in the synaptosomal (SYNP) and free mitochondrial (FBM) fractions of young (Y; 5 weeks old) adult (A; 5 months old) and old (O; 23 months old) animals. (C) Equal mitochondrial content in the synaptosomal fractions of different age-groups was confirmed by quantification of VDAC1 levels. Bars represent mean $\pm$ S.D. of duplicate measurements from 3 independent samples. (a) Denotes significant differences versus young group: * $p \leq 0.05$; *** $p \leq 0.001$

Figure 6. UNG1 and APE levels are progressively reduced in the synaptosomal fraction during aging. Age-related decrease in the motor protein Kinesin
 UNG1 (A) APE (B) and Kinesin (C) levels were detected by western blotting in young (Y; 5 weeks old) adult (A; 5 months old) and old (O; 23 months old) animals. Bottom panels show representative western blots, bands correspond to independent extracts. Equal mitochondrial content was confirmed by VDAC1 detection. Kinesin levels were normalized with actin levels. Bars represent mean $\pm$ S.D. of at least 3 independent samples. (a) Denotes significant differences versus young group; (b) denotes significant differences versus adult group: * $p \leq 0.05$; ** $p \leq 0.01$

A

B

C

D

Figure 7. BER activities in 3xTgAD mice are not changed in the synaptosomal fraction of whole brains

(A) 8-oxoG incision, (B) 5-OHC incision, (C) AP-site incision and (D) Gap filling activities in the synaptosomal fraction of 3xTgAD and control mice of 3 and 12 months-old. Bars represent mean $\pm$ S.D. of duplicate measurements from 3 independent samples. No statistical significant differences were found between groups.

TABLE 1

Oligonucleotides used in assays for DNA repair activities

Name	bp	Sequence
dU-BER	30	5'-ATA TAC CAC GTC GGU GAT CCA GTC CTG CTT 3'-TAT ATG GTG CAG CCG CTA GGT CAG GAC GTT
8oxoG	28	5'-GAA CGA CTG T8 A CTT GAC TGC TAC TGA T 3'-CTT GCT GAC ACT GAA CTG ACG ATG ACT A
dUracil	30	5'-ATA TAC CGC GUC CGG CCG ATC AAG CTT ATT 3'-TAT ATG GCG CGG GCC GGC TAG TTC GAA TAA
5-OHC	30	5'-ATA TAC CGC C5 C CCG GCC GAT CAA GCT TAT 3'-TAT ATG GCG GGG GGC CGG CTA GTT CGA ATA
AP	30	5'-ATA TAC CGC GGF CGG CCG ATC AAG CTT ATT 3'-TAT ATG GCG CCG GCC GGC TAG TTC GAA TAA
Gap-filling	34	5'-CTG CAG CTG ATG CGC XGT ACG GAT CCC CGG GTA C 3'-GAC GTC GAC TAC GCG GCA TGC CTA GGG GCC CAT G

The modified bases are shown in bold letters. Underlined bases indicate unpaired area, bp: base pairs.; **U** = dU; **8** = 8oxodG; **5**= 5-OHC; **X**= gap; **F** = tetrahydrofuran.