Rapid Communication • DOI: 10.2478/s13380-011-0011-8 • Translational Neuroscience • 2(1) • 2011 • 1-5

Translational Neuroscience

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MITOCHONDRIAL BIOENERGETICS IS DEFECTIVE IN PRESYMPTOMATIC TG2576 AD MICE

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Abstract

Alzheimer's disease (AD) is an age-related dementia, with the pathological hallmarks of neuritic plaques and neurofibrillary tangles, brain atrophy and loss of synaptic terminals. Dysfunctional mitochondrial bioenergetics is implicated as a contributing factor to the cognitive decline observed in AD. We hypothesized that, in the presence of the AD neurotoxic peptide beta-amyloid, mitochondrial respiration is impaired early in synaptic terminals, which are vital to cognitive performance, preferentially in cognitive centers of the brain. We compared oxygen consumption in synaptosomal and perikaryal mitochondria prepared from the cerebral cortex and cerebellum of wild type (WT) and AD transgenic Tg2576 mice. Compared to WT mice, Tg2576 mice showed decreased mitochondrial respiration in the cerebral cortex specifically in synaptosomal fraction, while the perikaryal mitochondria were unaffected. Neither mitochondrial fraction was affected in the cerebellum of Tg2576 mice as compared to WT. The occurrence of a bioenergetic defect in synaptic terminals of mice over-expressing mutant beta-amyloid, in particular in an area of the brain important to cognition, points to an early role of mitochondrial defects in the onset of cognitive deficits in AD.

Keywords

• Alzheimer's disease • Amyloid • Dementia • Energy Metabolism • Memory • Mitochondria • Oxidative phosphorylation • Oxygen Consumption • Tg2576

Received 15 March 2011
accepted 15 March 2011

Brain imaging studies have shown region-specific decrease in glucose utilization in Alzheimer's disease (AD) as compared to healthy brains [1,2]. Lower activities of the mitochondrial tricarboxylic acid cycle enzymes, alpha-ketoglutarate dehydrogenase complex [3,4] and pyruvate dehydrogenase [5,6] and of the electron transport chain complex cytochrome c oxidase or complex IV [7-10] have been reported in AD brains. These findings have led to the hypothesis that defective mitochondrial metabolism may be a cause of AD neurodegeneration [11].

Recent studies reported that brain mitochondrial function declines in a triple transgenic AD mouse model prior to the onset of amyloid plaque pathology [12] and that synaptic mitochondria are more affected than non-synaptic mitochondria in cognitively impaired AD transgenic mice [13]. We hypothesized that the early bioenergetic defect may develop preferentially in cognitive areas of the brain, specifically in synaptic mitochondria, in cognitively normal AD mice. To test this hypothesis, we used the Tg2576 mouse model of AD, which expresses human amyloid precursor protein (APP) with the Swedish double mutation K670N, M671L. This model is characterized by the onset of cognitive decline by the age of 9 months, associated with increased levels of beta-amyloid (Aβ) peptides in the brain [14].

For preparation of synaptosomal and perikaryal mitochondria, cerebral cortex and cerebellum from female C57B6/SJL wild-type (WT, n=20, 5-15 month old) and pre-symptomatic Tg2576 mice (n=10, 5-8 month old) were homogenized in STE buffer (250 mM sucrose, 5 mM Tris, 2 mM EGTA, pH 7.2, with 0.2% fatty acid free bovine serum albumin, BSA) using a Precellys24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5,500 rpm for 20 s. Two samples of each group were pooled, layered on a discontinuous Percoll gradient as per the method of [15] and centrifuged at 30,400 x *g* for 30 min at 4°C in the SW41Ti swing-out rotor of a Beckman L7-55 centrifuge (Beckman Coulter Inc., Brea, CA, USA). The synaptosomes and perikaryal mitochondria were washed once in STE buffer, followed by two washes in STE buffer without BSA and protein estimated by the Bradford assay.

For analysis of respiration, synaptic terminals were resuspended in KCl respiration buffer (125 mM KCl, 0.1% BSA, 20 mM HEPES, 2 mM $MgCl$, 2.5 mM KH_2PO_4, pH 7.2) and attached to the Seahorse V7 cell plate at 10 µg protein per well by centrifugation at 3,220 x g for 1 h at 4°C. Oxygen consumption rate (OCR) in synaptic terminals was measured using the Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA, USA) essentially by the method of [16].

The perikaryal mitochondria were seeded at 5 µg per well onto the Seahorse V7 cell plate in mitochondrial assay solution (MAS; 70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1 mM EGTA, 0.2% fatty acid free BSA, pH 7.2) and centrifuged at 2,000 *x g* for 10 min at 4°C. The assay was programmed for the following steps: 35 min

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calibration; 12 min equilibration; two cycles of 30 s mix and 4 min measure for basal OCR; 15 s mix, injection of ADP, 15 s mix, 6 min measure; two repeats of 15 s mix, injection (oligomycin or FCCP), 15 s mix, 3 min measure and a final 15 s mix, injection of a mixture of rotenone, antimycin A, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) and ascorbate, followed by a 15 s mix and 2 min measure for complex IV OCR.

As markers for synaptosomes and mitochondria, synaptophysin and voltage dependent anion selective channel protein 1 (VDAC1) were identified by Western blot and band intensities were quantified using ImageJ 1.43u software (National Institutes of Health, USA).

For quantifying the levels of soluble oligomeric Aβ, samples were prepared as previously described [17] and assayed by Western blots using mouse monoclonal antibody clone 4G8 against APP (Signet, USA), with actin as loading control. Total Aβ peptides in the Tg2576 brain were measured by ELISA using human $Aβ_{1-40}$ and $Aβ_{1-42}$ kits (Invitrogen, Camarillo, CA).

Data were analyzed using Prism 4.02 software (GraphPad Software Inc., La Jolla, CA, USA). The difference between groups was analyzed using two-way analysis of variance (ANOVA) or two-tailed *t* test, with *p* values less than 0.05 considered significant.

The sites of action of the substrates, inhibitors and mitochondrial uncoupler used in the measurement of OCR using the Seahorse technology are illustrated in Figure 1. Briefly, mitochondria are provided with pyruvate and malate as substrates for the measurement of basal OCR. Then, excess ADP is injected into the wells and the ADP-stimulated OCR is measured. The addition of oligomycin inhibits complex V, the ATPase, so that phosphorylation is absent, the protons pumped by the electron transport chain build up in the mitochondrial inter-membrane space and the OCR is reduced. The mitochondrial uncoupler FCCP releases the proton gradient and enables maximal OCR. In the last stage of the analysis, complexes I and III are inhibited by rotenone and antimycin respectively and ascorbate and TMPD are provided as artificial electron donors to measure complex IV activity.

To establish whether synaptic mitochondria show bioenergetic deficits prior to cognitive impairment, we analyzed mitochondrial respiration in the cerebral cortex of pre-symptomatic Tg2576 mice compared to WT controls. Since no significant age-related change was observed in WT mice of two age groups (5-8 and 13-15 month old, data not shown), we combined the data from these two groups as control. We found a significant decrease in basal OCR (mean ± SEM for Tg2576 *vs* WT = 33.3 ± 4.4 *vs* 43.5 ± 3.4 pmol oxygen/min), maximal OCR (mean ± SEM for Tg2576 *vs* WT = 30.7 ± 1.8 *vs* 61.2 ± 6.3) and complex IV OCR (mean ± SEM for Tg2576 *vs* WT = 107.3 ± 13.5 *vs* 142.5 ± 17.9) of cortical synaptosomal fraction of Tg2576 mice as compared to the WT controls (Figure 2A, $p < 0.0001$ by two-way ANOVA). However, in perikaryal fraction mitochondria of the cortex, there was no significant difference in OCR between Tg2576 and WT (Figure 2B).

In order to ensure that the observed changes in mitochondrial respiration were not due to altered mitochondrial number between Tg2576 and WT, we estimated the levels of a mitochondrial marker protein, VDAC1. VDAC1 expression in both the synaptosomal (Figure 3A) and perikaryal fractions (Figure 3B) were unchanged, indicating that the observed changes in oxygen consumption were not due to differences in the mitochondrial content. We also confirmed that the amount of synaptosomes from the two groups of animals were comparable by quantifying the synaptic protein, synaptophysin, in the synaptosomal fraction (mean ± SEM of band optical densities were 14748 ± 733.5 and 16630 ± 1332 respectively).

Since the synaptosomal bioenergetic defect we found occurred in the cerebral cortex, an area of the brain that is important to cognition, we evaluated whether this effect might be specific to cognitive areas of the brain. Towards this end, we analyzed OCRs in the cerebellum and found no change between the WT and AD mice in the basal (Figure 4A,D), maximal (Figure 4B,E) or complex IV OCR (Figure 4C,F) in either synaptosomal (Figure 4A-C) or perikaryal fraction mitochondria (Figure 4D-F).

As mitochondrial dysfunction has been reported in the presence of Aβ, we assessed the levels of Aβ in Tg2576 mice. Soluble oligomeric Aβ of 56 kDa and 100 kDa (Figure 5A) and total $Aβ_{1-40}$ and $Aβ_{1-42}$ peptides were present in detectable levels in Tg2576 brains (Figure 5B).

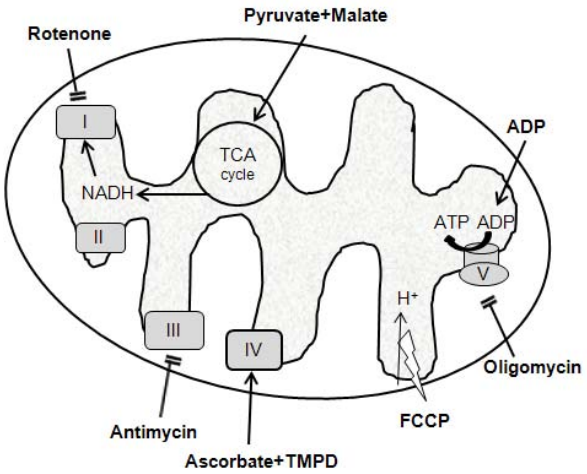

Figure 1. **Oxygen consumption rates (OCR) in mitochondria.**
Schematic figure showing the substrates, uncoupler and inhibitors used in the OCR analysis and the steps of the oxidative phosphorylation pathway where they act. Clockwise from the top: pyruvate and malate enter the tricarboxylic acid (TCA) cycle and provide NADH to complex I of the electron transport chain; ADP provides the substrate for phosphorylation at complex V; oligomycin inhibits complex V and thence oxidation; p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) uncouples oxidation from phosphorylation by releasing the proton gradient across the mitochondrial inner membrane and leading to maximal OCR; ascorbate and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) act as artificial electron donors for complex IV; antimycin and rotenone inhibit complexes III and I respectively.

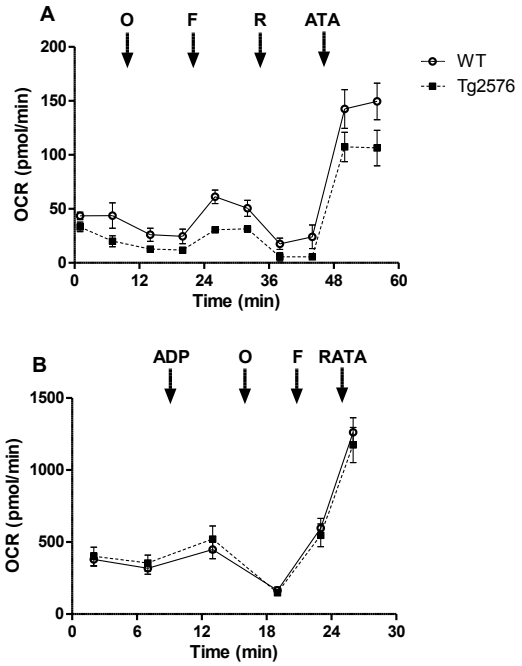

Figure 2. **Oxygen consumption rates (OCR) in cerebral cortex of wild type (WT) and Tg2576 mice.** Oxygen consumption rates in **(A)** synaptosomal fraction and **(B)** perikaryal fraction mitochondria from the cerebral cortex of WT and Tg2576 mice measured using the Seahorse extracellular flux analyzer. Basal, adenosine diphosphate (ADP)-stimulated, oligomycin-inhibited, p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) induced maximal and complex IV OCRs were measured as pmol O_2/min using the Seahorse XF24 extracellular flux analyzer. The incubation medium for synaptosomes contained 10 mM pyruvate and four sequential injections delivered 16 µM oligomycin (O), 4 µM FCCP (F), 4 µM rotenone (R) and a mixture consisting of 1 µM antimycin A, 12.5 µM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) and 1.25 mM ascorbate (ATA). For perikaryal mitochondria, the assay medium contained 10 mM each of pyruvate and malate and the injections consisted of 1 mM ADP, 2 µM oligomycin, 8 µM FCCP and a mixture of 2 µM rotenone, 4 µM antimycin A, 50 µM TMPD and 5 mM ascorbate (RATA). Data are mean ± SEM of n=6 for WT and n=3 for Tg2576 synaptosomal fraction; n=10 for WT and n=5 for Tg2576 perikaryal fraction. $P < 0.0001$ when comparing Tg2576 to WT synaptosomal fraction by two-way ANOVA.

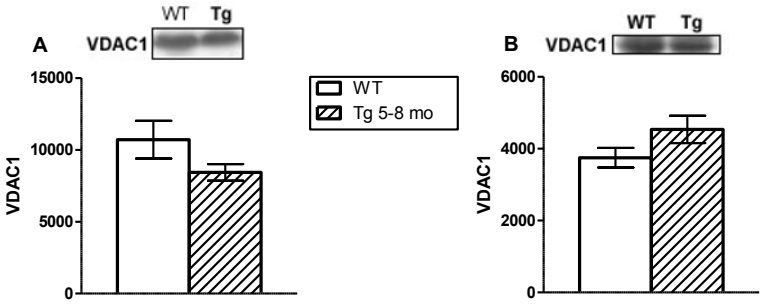

Figure 3. **Expression of mitochondrial marker proteins in cerebral cortex of wild type (WT) and Tg2576 mice.** Expression level of VDAC1 in **(A)** synaptosomal fractions and **(B)** perikaryal mitochondria prepared from cerebral cortex of WT and Tg2576 mice were examined by Western blots using anti-VDAC1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Data are represented as mean ± SEM of 3-7 replicates in arbitrary units (AU). The insets show representative Western blots for VDAC1. No significant difference was detected as assessed by two-tailed *t* test.

Our findings corroborate a recent report on early synaptic mitochondrial dysfunction in the cortex of a Tg mAPP (V717F, K670M, N671L mutant of human APP) mouse model of AD [18]. Additionally we show the presence of the synaptic mitochondrial defect specifically in the cerebral cortex, but not the cerebellum, prior to the onset of cognitive deficits in Tg2576 mice [14]. While an earlier study had shown a synaptic bioenergetic deficit in the cortex, hippocampus, striatum and amygdala, but not the cerebellum, of Tg2576 mice at 12 months of age [13], our results demonstrate that the synaptic mitochondrial defect is manifest at an age when the mice are reported cognitively intact.Co-localization of Aβ [19] and APP [20] with brain mitochondria has been reported in AD and in Tg2576 [21,22]. Both $Aβ_{1-40}$ and $Aβ_{1-42}$ forms were found in mitochondria from Tg2576 cortex [22], with preferential Aβ accumulation in the synaptic mitochondria of 12 month old Tg mAPP mice [18]. $Aβ_{1-42}$ in the presence of copper specifically inhibited complex IV activity in isolated mitochondria [21]. While amyloid plaques are not detected in 5-8 month old Tg2576, soluble forms of Aβ, such as the oligomers found in our study, may inhibit mitochondrial respiration in synapses and set in motion the loss of synapses and subsequent cognitive decline observed at a later age [23].

Our results in Tg2576 mice indicate that bioenergetic deficits in the synaptic terminals in cognitive centers of the brain precede and may contribute to the onset of cognitive dysfunction in AD, providing a target for early therapy.

Competing interests:

The authors declare that they have no competing interests.

Author contributions:

MV conceived the project, designed and executed the experiments for mitochondrial function, analyzed the data and drafted the manuscript. WZ participated in synaptosomal and perikaryal fraction sample preparation and analysis and manuscript correction. JW

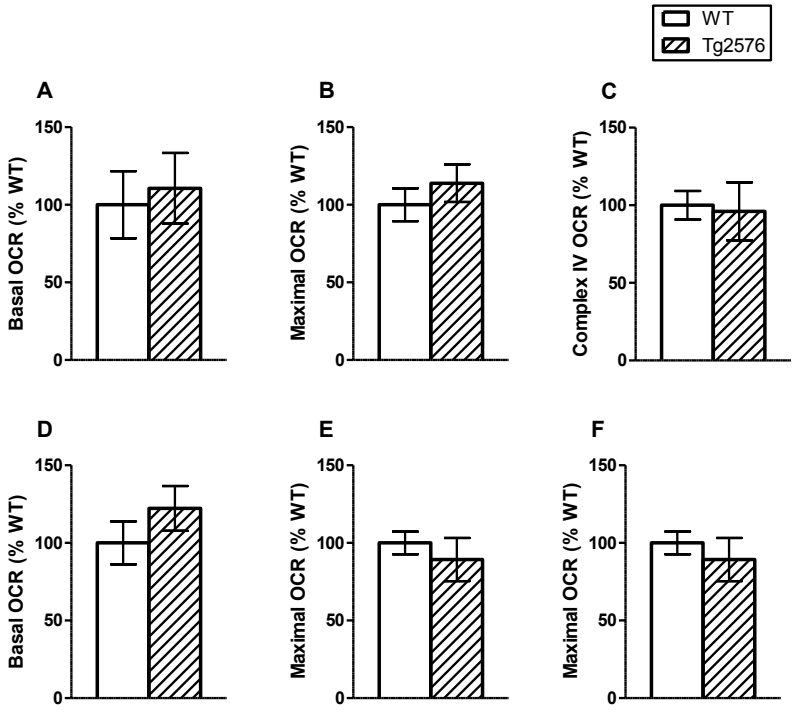

Figure 4. **Oxygen consumption rates (OCR) in cerebellum of wild type (WT) and Tg2576 mice.**
(A) Basal, **(B)** maximal and **(C)** complex IV OCR in synaptosomal fraction and **(D)** basal, **(E)** maximal and **(F)** complex IV OCR in perikaryal fraction mitochondria from the cerebellum of WT and Tg2576 mice measured using the Seahorse extracellular flux analyzer. Data are expressed as percentage of OCR in the WT mice (mean ± SEM, n=8 for WT and n=4 for Tg2576). No significant difference was observed as assessed by two-tailed *t* test.

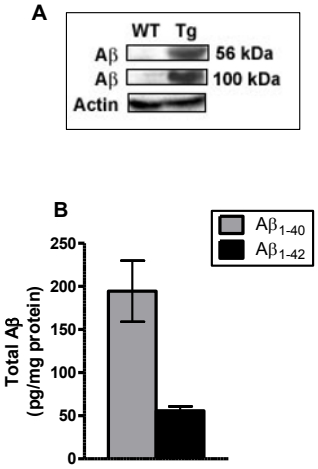

Figure 5. **Beta-amyloid (Aβ) levels in the brain of Tg2576 mice.**
(A) Soluble oligomeric Aβ and actin levels in WT and Tg2576 mice. Tg2576 brain supernatants were immunoprecipitated using mouse anti-APP A4 antibody (clone 22C11, Chemicon, Millipore, Billerica, MA, USA) to remove full length APP and Aβ oligomers were detected by Western blot using anti-APP clone 4G8. **(B)** Total $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptide levels in Tg2576 mice measured by ELISA. The brain homogenates in STE buffer were extracted 1:4 (v/v) with formic acid overnight, neutralized with 1:9 (v/v) 2 M Tris-HCl (pH 11) containing protease inhibitors, centrifuged at 16,000 *x g* for 5 min and the supernatants used for ELISA. Data are expressed as pg Aβ/mg protein (mean ± SEM, n = 8-11).

participated in beta-amyloid quantification, data interpretation and in structuring the draft. AC carried out tissue dissections and assisted in sample preparation. XQ carried out animal maintenance. AC conducted the Western blots. LH participated in study design and co-ordination, data interpretation and manuscript preparation. GMP discussed the concept, supervised the project and contributed to data presentation. All authors read and approved the final manuscript.

Acknowledgments:

We are grateful to Dr David B. Hicks for technical assistance. This project was funded by grants from the National Institutes of Health (NIH AG02219 Project 2) and the Department of Veterans Affairs.

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